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(54) Title: THE MAST CELL SECRETORY MACHINE AS A TARGET FOR ANTI-ALLERGY DRUG DEVELOPMENT

(57) Abstract

The present invention relates to a method for the development of drugs that impair stimulated secretion from mast cells by blocking the function of one or more components of the exocytic mechanism. This method depends upon identification of the molecular components of the mast cell exocytic mechanism. Such knowledge is then used to rationally design molecules intended to block productive interactions of these components, followed by assay of the efficacy of the rationally designed molecules in an *in vitro* assay of protein–protein interaction and/or a cellular assay of mast cell secretion. Alternatively, an *in vitro* assay of interactions among components of the exocytic mechanism is used to empirically identify molecules that disrupt these interactions, this can then be followed by an assay of efficacy in intact mast cells. These methods provide a means of identifying molecules that impair mast cell secretion. Such molecules could be useful drugs in the prevention and/or treatment of allergic reactions, including but not limited to hay fever, asthma, eczema, urticaria, and anaphylaxis.

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The Mast Cell Secretory Machine as a Target For Anti-allergy Drug Development

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Field of the Invention

The present invention relates to a method for the identification of molecules that are useful as drugs to prevent allergic inflammation. More particularly, the process relates to drugs which inhibit secretion from mast cells by impairing function of the exocytic mechanism.

Background of the Invention

Allergic diseases are a major source of morbidity in the United States. Approximately 35% of the American population suffers from allergic rhinitis (also called "hay fever"), 5% from asthma, 15% from urticaria, 15% from eczema, and 1% from anaphylaxis. These disorders can cause lost work and an impaired quality of life, and in the case of asthma and anaphylaxis, can be fatal. Mast cell activation is the central event in allergic inflammation. Mast cells are most commonly activated by the binding of antigens (also called "allergens") to IgE molecules bound to the mast cell surface. They may also be activated by other stimuli such as physical forces or ligands such as substance P. Upon activation, mast cells respond through three principal mechanisms: 1) the exocytosis of preformed inflammatory mediators from secretory granules, 2) the *de novo* generation of lipid mediators, including prostaglandins, leukotrienes, and platelet activating factor, and 3) the generation of cytokines that recruit other inflammatory cells

such as eosinophils, neutrophils, and lymphocytes, which then perpetuate the inflammatory reaction.

The exocytosis of preformed mediators from mast cells is particularly important in the acute phase of allergic reactions. These preformed mediators include biogenic amines such as histamine, a variety of proteases capable of degrading extracellular matrices and of activating other mediators, and proteoglycans such as heparin. Most current therapies of allergic reactions focus on blocking the body's response to secreted mediators, such as the use of antihistamines to block the itching and swelling caused by histamine in hay fever and urticaria; the use of α-adrenergic agonists in the nasal passages to reduce swelling caused by histamine and other mediators in hay fever; the use of β-agonists in the airway to reduce bronchoconstriction caused by mediators in asthma. However, these therapies treat only one target of mast cell mediators, and have only a small impact in reducing the ongoing inflammatory reaction. Cromolyn sodium is a broader based medication; it is a compound that was empirically found to reduce mast cell secretion, and is now thought to work by inactivating chloride channels. However, it has low efficacy. Glucocorticosteroids are efficacious anti-inflammatory agents, but have serious side effects such as growth retardation and reduction of bone mineralization.

In contrast to previously available therapies, compounds that specifically impair mast cell exocytosis are of low toxicity because regulated exocytosis is not a vital function for mast cells, leaving them available to participate in other aspects of immune function. In human beings, mast cell exocytosis has little or no utility in persons living in developed countries. Mast cell activation appears to have evolved primarily to function in defense against helminthic infestation. Such

infestations are rare in Western societies, and mast cell degranulation is now observed almost exclusively as part of dysfunctional allergic reactions. Therefore, blockage of mast cell exocytosis *per se* is effective in preventing allergic inflammation and has few undesirable consequences.

Despite intense study of the nature of allergic responses, the molecular mechanism of mast cell exocytosis remained poorly understood. Recent progress in elucidating the molecular composition and mechanism of vesicular transport processes in organisms as diverse as yeast and mammals indicates that conserved families of proteins play similar roles in most organisms and most transport steps. For example, Syntaxin1A is a plasma membrane protein that mediates exocytic fusion in neurons, the homologs Sso1 and Sso2 play similar roles in yeast exocytosis, and the Sed5 homolog functions in endoplasmic reticulum to Golgi transport in yeast. Different isoforms (or isotypes) of exocytic proteins are often found in different mammalian cell types. For example, Rab3A regulates neuronal exocytosis, whereas Rab3B plays a similar role in pituitary cells and Rab3D in mast cells. Presumably, the unique properties of each isoform underlie differences in secretory mechanisms in different cell types, such as the millisecond responsiveness of neurons contrasted with a several minute duration of exocytosis in mast cells. In addition to the proteins conserved between yeast and man, some proteins such as the calcium sensor Synaptotagmin are found only in higher eukaryotes. Together, these data suggest that individual isoforms of conserved components of vesicle transport machines can be found associated with a particular transport process such as mast cell exocytosis, along with some components that are less widely expressed.

Summary of the Invention

An object of the present invention is a method of identifying the molecular components of the mechanism that mediates mast cell exocytosis.

Another object of the invention is to enable the rational design of compounds that exhibit agonistic or antagonistic effects on the mast cell secretory machine.

A further object of the invention is to identify compounds that alter interactions of the components of the mast cell secretory machine.

A further object of the invention is to measure the extent of the ability of compounds to alter interactions of the components of the mast cell secretory machine.

Another object of the invention is to measure the efficacy of compounds in impairing stimulated mast cell exocytosis of preformed granules using mast cell secretion assays.

An additional object of the invention is a gene therapy method for altering exocytic protein production in mast cells.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method of identifying the molecular components of the mast cell secretory machine comprising the steps of: quantitatively measuring the gene expression in a mast cell or mast cell line by measuring a protein or mRNA product; locating the subcellular site of the protein product; and determining whether such product is a component of the mast cell secretory machine.

Specific embodiments of the invention include an RNAse protection assay, a Northern blot assay, a quantitative reverse-transcription polymerase chain reaction (RT-PCR) assay, or a quantitative immunoblotting assay to determine quantitative gene expression.

Another specific embodiment of the present invention includes the use of immunofluorescence microscopy to determine subcellular localization of the mast cell secretory machine component.

Also, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention a method for identifying compounds exhibiting an agonistic or antagonistic effect on the mast cell secretory machine comprising of: identifying the component of the mast cell secretory machine and screening for compounds that exhibit agonistic or antagonistic effects with said product by testing the compound's activity in the mast cell secretory machine.

Specific embodiments of the invention include, but are not limited to proteins such as Rab3, Rab3D, Synaptotagmin, Synaptotagmin2, Synaptotagmin3, Syntaxin, Rabphilin, Cellubrevin/VAMP, SEC1/Rop/Munc18, Sec1-B, SNAP-25, Cysteine string protein, Complexin, SCAMP, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, NSF, or $\alpha/\beta/\gamma$-SNAP as mast cell secretory machine components.

Other specific embodiments include an assay of protein-protein interactions, or an assay measuring the degree of effect on protein-protein interactions.

Also in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method of quantifying the effect of a compound identified as a component of the mast cell secretory machine comprising an assay determining the amount of degranulation of mast cells.

A further embodiment of the present invention is a method of gene therapy for altering mast cell secretion comprising the step of transducing bone marrow stem cells with a gene construct designed to promote expression of exocytic proteins in mast cells.

In specific embodiments the stem cells are transduced either *ex vivo.* or *in vivo.*

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention which are given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows β-glucuronidase release from antigen-stimulated RBL cells. A representative example of low passage-number RBL cells stimulated to secrete β-glucuronidase by cross-linking IgE receptors with antigen and antigen-specific IgE. Lane 1- Triton X-100 total cell lysate; 2 - control culture supernatant of cells exposed to DNP-BSA alone; 3 - control culture supernatant of cells exposed to DNP-specific IgE alone; 4 - culture supernatant of cells exposed to both IgE and DNP-BSA.

Figure 2 shows the alignment of Rab3 isoforms. The inferred amino acid sequences of all known mammalian isoforms of Rab3 are

aligned with H-Ras. Accession numbers are listed after each protein's name, conserved G-domains are shaded, the regions corresponding to the Rab3-specific PCR primers used herein are double-underlined, the peptide antigen used for the Rab3D-specific antiserum is shown in outline, and the mapped epitope of pan-Rab3 monoclonal antibody 42.1 is underlined. The rat Rab3B sequence is a composite of a previously cloned fragment and the PCR product reported in this manuscript. The rat Rab3D sequence was originally reported as Rab16, but is 98% identical to mouse Rab3D when corrected for two sequencing errors that resulted in a local frame shift encompassing 12 amino acids (a missed guanine in the first position of the codon for alanine 97 and an extra cytosine in the codon for glutamine 110) .

Figure 3 is a Northern blot of Rab3 isoforms in RBL cells. A 385 nucleotide Rab3B coding region riboprobe was hybridized with 4 μg each of poly(A)$^+$ RNA from rat brain, rat lung, and RBL cells. An autoradiogram is shown, with size markers on the left side and the reported sizes of Rab3 transcripts on the right side.

Figure 4 shows an RNase protection assay of RBL cell transcripts. Autoradiogram of the products of an RNase protection assay following polyacrylamide gel electrophoresis. First four lanes - 8 μg each of poly(A)$^+$ RNA, fifth lane - 4 μg, sixth lane - 1.6 μg. The radiolabeled riboprobes used for hybridization in each lane are listed in the top row. Shown is a representative example of an assay performed four times

Figure 5 is an immunoblot of RBL proteins using a pan-Rab3 antibody. Monoclonal antibody 42.1 was used to probe Western-blotted proteins. First lane - 2.5 ng purified Rab3A, second lane - 30 μg pancreatic homogenate, third lane - 5 ng purified Rab5A, fourth lane - 3

μg brain homogenate, fifth lane - 100 μg RBL cell lysate, sixth lane - 3 μg Rab3D-expressing *E. coli* lysate. The migration of molecular weight standards, listed in kDa, is plotted on the left.

Figure 6 is an immunoblot of RBL proteins using a Rab3D-specific antiserum. Affinity purified Rab3D antiserum was used to probe Western-blotted proteins. First lane - 5 ng purified Rab3A, second lane - 15 μg pancreatic homogenate, third lane - 5 ng purified Rab5A, fourth lane - 100 μg brain homogenate, fifth lane - 100 μg RBL cell lysate, sixth lane - 15 μg Rab3D-expressing *E. coli* lysate. The migration of molecular weight standards, listed in kDa, is plotted on the left. The migration of Rab3D from RBL cells determined using monoclonal antibody 42.1 is indicated by the arrow on the right.

Figure 7 shows the quantification of Rab3D protein expression in RBL cells. Fig. 7A The immunoreactivity of 25 μg of RBL cell lysate (open circle) and increasing amounts of GST-Rab3D (filled circles) were compared by Western blot analysis using Rab3D-specific antiserum. The mass of GST-Rab3D is expressed as ng of Rab3D protein. The 50 kDa marker on the right correspond to GST-Rab3D and the 24 kDa marker to Rab3D from RBL cells. Fig. 7B Plot of the results of densitometric scanning of the immunoblot in "A".

Figure 8 shows the immunocytochemical localization of Rab3D and RBL cell granule markers. Cells were imaged by indirect immunofluorescence using primary antibodies specific for the proteins indicated and FITC-conjugated secondary antibodies. Fig.8A AD1, Fig. 8B RMCP-II, Fig. 8C pan-Rab3 (polyclonal antiserum), Fig. 8D Rab3D.

Figure 9 shows the colocalization of Rab3D and RMCP-II in RBL cells.

Figure 10 shows the localization of Rab3D in peritoneal mast cells and in activated RBL cells. Rat peritoneal mast cells were allowed to air dry on glass slides, then labeled with antibodies to Rab3D and imaged by phase contrast microscopy Fig. 10A or by indirect immunofluorescent microscopy using Texas Red-conjugated secondary antibodies Fig. 10B. RBL cells cultured on coverslips were activated for secretion by cross-linking bound IgE with specific antigen Fig. 10C, as in Fig. 2. After 15 min they were fixed, labeled with antibodies to Rab3D, then imaged using Texas Red-conjugated secondary antibodies.

Figure 11 shows a model of the regulation of mast cell exocytosis by GTPases. The shaded box represents the fusion machine itself, and G_E is the target of GTPγS that promotes exocytosis in patch clamped mast cells. Unbroken lines represent activities that are resistant to washing out by a patch pipette, and broken lines represent activities that are washed out. Arrows represent stimulatory activities, and perpendicular dashes represent inhibitory activities.

Figure 12 shows the predicted 365 bp Syt product of nested PCR reactions.

Figure 13 shows Syt isoforms 2, 3, and 5 identified by sequencing subclones from the product of the nested PCR reactions.

Figure 14 shows an autoradiogram demonstrating that the Syt2 probe is best protected by RBL cell mRNA in an RNAse protection assay, followed by Syt3.

Figure 15 shows colocalization of Rab3D and secretory granule markers in RBL cells and mast cells. RBL cells were labeled with antibodies to RMCP-II Fig. 15B, mast cells were labeled with FITC-

avidin Fig. 15E, and both cell types were labeled with antibodies to Rab3D Figs.15A and 15D. Antibodies were then imaged by indirect immunofluorescence laser confocal microscopy using secondary antibodies labeled with Texas Red Figs.15A and 15D or FITC Fig. 15B. FITC-avidin Fig. 15E was imaged directly. In the computer-merged images Figs. 15C and 15F, yellow color indicates coincident red and green fluorescence.

Figure 16 shows FISH analysis of mapping experiments.

Figure 17 is a schematic representation of a gene structure for altering Rab3D expression.

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Detailed Description of the Invention

It is readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

"Exocytosis" refers to the process whereby a membrane-delimited secretory vesicle fuses with the plasma membrane, releasing the soluble contents of the vesicle into the extracellular space and inserting the membranous components of the vesicle into the plasma membrane. Herein, "exocytosis" generally refers to the process of regulated (or stimulated) exocytosis, whereby vesicles formed in the Golgi apparatus fuse with each other to form large granules and then await a signal before undergoing fusion with the plasma membrane.

This contrasts with constitutive exocytosis, whereby small secretory vesicles formed in the Golgi apparatus travel to the plasma membrane for fusion without waiting for a signal.

"Secretory granules" or "granules" refer to the large post-Golgi secretory vesicles of mast cells that contain inflammatory mediators such as histamine, proteases, and proteoglycans.

"Antagonistic effect" refers to the response of mast cells to compounds that inhibit or prevent secretion by mast cells. Such "antagonistic" compounds inhibit the release of inflammatory mediators from mast cells.

"Agonistic effect" refers to the response of mast cells to compounds that stimulate or activate secretion by mast cells. Such "agonistic" compounds initiate or increase the amount of secretion from mast cells.

In one embodiment of the present invention there is a method of identifying molecular components of the mast cell secretory machine comprising the steps of: quantitatively measuring the gene expression in a mast cell or mast cell line by measuring a protein or mRNA product; locating the subcellular site of the protein product; and determining whether such product is a component of the mast cell secretory machine.

In one preferred embodiment of the present invention PCR is used, although any process of DNA amplification can be used. Oligonucleotide primers are designed to amplify all members of a particular protein family (e.g. Rab proteins) expressed in mast cells or a mast cell line (e.g. RBL-2H3). Family members are selected on the

basis of conserved sequences. A subset of the family is amplified if that subset is known to regulate exocytosis (e.g. the Rab3 subset of proteins; A, B, C, and D). Mast cell mRNA or mRNA from a mast cell line is reverse transcribed into cDNA, then amplified using the specific primers. The PCR product is then subcloned, and individual colonies sequenced until it appears that all amplified DNA is represented (i.e. the same ones keep turning up, typically 15 to 30 colonies). Restriction analysis of the pooled PCR product indicates whether certain species are abundantly present or not, as in the Rab3 and Synaptotagmin examples. The amplified DNA now serves as templates for generating riboprobes or DNA probes for quantitative expression analysis, such as by RNase protection assay. This process identifies the major species of a particular family of exocytic genes expressed in mast cells at the RNA level.

In other embodiments of the present invention, the relative expression of protein products is quantitated using isoform-specific antipeptide sera and quantitative immunoblotting, Northern blot analysis, or some other comparable immunologic technique. In another embodiment of the present invention, the quantitative gene expression is determined by an RNAse protection assay. A further embodiment of the present invention includes a quantitative reverse-transcription polymerase chain reaction (RT-PCR) assay.

In one embodiment of the present invention, the subcellular localization of the mast cell secretory machine component involves immunofluorescence microscopy. This technique provides further evidence of a particular protein's participation in mast cell exocytosis by localizing the protein to a predicted compartment. For example, SNAP-25 and Syntaxin are found in target membranes for fusion (in this case the plasma membrane), whereas in the resting state

Synaptotagmins are found in donor membranes (in this case the secretory granule membrane).

One embodiment of the present invention includes a method for identifying compounds exhibiting an agonistic or antagonistic effect on the mast cell secretory machine comprising of: identifying the product of the mast cell secretory machine; and screening for compounds that exhibit agonistic or antagonistic effects with said product by testing the compound's activity in the mast cell secretory machine.

Various embodiments of the present invention include, but are not limited to proteins such as Rab3, Rab3D, Synaptotagmin, Synaptotagmin2, Synaptotagmin3, Syntaxin, Rabphilin, Cellubrevin/VAMP, SEC1/Rop/Munc18, Sec1-B, SNAP-25, Cysteine string protein, Complexin, SCAMP, Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, NSF, or $\alpha/\beta/\gamma$-SNAP as components.

New drugs are identified based upon all of the above data and analogy with the known functions of closely related isoforms in other cell types. Alternatively, the protein components involved in mast cell exocytosis are identified by overexpressing wild-type and/or mutant protein in a mast cell line and measuring changes in secretion. In another approach, knockout mice are generated in which the gene of interest is deleted, and the secretory function of mast cells harvested from the peritoneum is measured in a high resolution exocytosis assay. These functional studies are used to identify particular proteins involved in mediating mast cell exocytosis.

Embodiments of the present invention include methods wherein the screening comprises an assay of protein-protein interactions, or

comprises an assay measuring the degree of effect on protein-protein interactions.

The three dimensional structures obtained by crystallography, NMR spectroscopy, or other physical techniques are used to assist in the rational design of compounds that exhibit agonistic or antagonistic effects. Such structures are often available for another member of the family, and a predicted structure of the isoform of interest is obtained by homology modeling. Also, knowledge of the regions of interaction of two components of the exocytic mechanism is used to aid drug design. A higher degree of specificity is provided in designing a drug because even if the mast cell secretory machine shares a component with another exocytic mechanism, they may have different partners. The information accumulated in the literature of protein-protein interactions in vesicle transport mechanisms is used by homology. Direct information is obtained utilizing assays of protein-protein interaction such as plasmon resonance spectroscopy, or scintillation proximity assay. Mutagenesis of single amino acids or small regions in concert with the protein-protein assay is also used to pinpoint the interactions. The above information - the identity of the components, their tertiary structures, and their regions of interaction - is available for rational design of inhibitory molecules. All of the above information is derived directly from the method of the invention which supplies the basic sequential and structural information.

A further embodiment of the invention includes a method of quantifying the effect of a compound on the mast cell secretory machine comprising an assay determining the amount of degranulation of mast cells.

The activities of rationally designed molecules are tested *in vitro* using a protein-protein assay as above, and/or a cellular exocytic assay as follows. Membrane-permeant small molecules or molecules designed to interact with the extracellular domains of exocytic proteins are introduced directly into cell cultures. Secretion is then triggered by the addition of IgE and specific antigen. The effect of the rationally designed inhibitory molecules is measured in a dose-response assay. For membrane-impermeant molecules targeted to the intracellular region of exocytic proteins, mast cells are permeabilized by the addition of a low concentration of a detergent, or similar technique. Molecules that have an effect are rationally modified to increase membrane permeability.

Preferred embodiments of this invention include secretion assays that contain permeabilized or intact mast cells, and use primary mast cells or mast cell lines (such as RBL-2H3).

In one embodiment, secretion-inhibitory molecules are empirically identified by screening libraries of compounds for disruption of the protein-protein interactions *in vitro*, as above, or for their ability to impair secretion in a cellular assay. The site of action of such compounds is then localized by systematically analyzing interactions of components of the exocytic machine in the presence and absence of the inhibitory molecule.

Use of the methods of the invention supplies predicted or actual amino acid sequences and enables prediction or actual identification of three dimensional structures of the components. A person skilled in the art can use the invention to design drug compounds based on the components identified by the invention.

A further embodiment of the present invention is a method of gene therapy for altering mast cell secretion comprising the step of transducing bone marrow stem cells with a gene construct designed to promote expression of exocytic proteins in mast cells. In specific embodiments the stem cells are transduced either *ex vivo.* or *in vivo.*

Alteration of production of exocytic proteins in mast cells can be used in the treatment of asthma, anaphylaxis, hay fever and other allergic conditions. It is also known that exocytic protein alteration may affect inflammation disorders such as those that lead to fibrosis. Mast cell secretion and exocytic protein production can be altered by genetic mutations or by addition of promoters. The increased production of the exocytic proteins appears to ameliorate the disease state.

Another specific embodiment is altering the level of expression of native components of the mast cell exocytic machine or of mutant forms of the components. An example of this the use of gene therapy. In certain cases, this is expected to suppress mast cell secretion. This is performed in mice for the purpose of studying alterations in mast cell physiology and/or whole animal physiology to model potential therapies, or it is performed in human beings for therapeutic purposes (i.e. gene therapy).

Another specific embodiment is mapping the genes of mast cell exocytic components in mice and human beings. In particular, the locations of these genes can be compared to genetic loci mapped through whole genome searches for asthma or allergy genes of mice and Human beings. With or without such supporting data, the structures of the genes encoding components of the mast cell exocytic machine can be analyzed for mutations that account for some of the variable genetic

susceptibility to asthma or allergy. This can be used in a diagnostic assay. In addition, the identification of specific mutations in individual patients can form the basis of individually tailored therapy either with small molecules or by gene therapy.

Another specific embodiment is expressing mammalian mast cell exocytic proteins in model lower organisms, including but not limited to the fruit fly Drosophila melanogaster or the budding yeast Saccharomyces cerevisiae, in order to screen compounds for their ability to alter mast cell secretion. This can be done either by expressing the mammalian protein on top of the endogenous proteins of the lower organism, or the mammalian protein may be substituted in deletants of the endogenous gene.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

EXAMPLE 1

Cell culture and measurement of secretion

RBL-2H3 cells (hereafter referred to as "RBL cells") were grown in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin and streptomycin. They were subcultured every 3-4 days when confluent. Their secretory phenotype was periodically assessed by measurement of antigen-induced β-glucuronidase release as follows. Forty-eight well plates were coated with fibronectin by the method of Hamaway et al., J. Immunol., Vol. 149, pp. 615-621 (1992), and ~400,000 RBL cells were aliquoted per well. Anti-dinitrophenol (DNP) IgE was then added to a final concentration of 1 μg/ml and the cells were incubated overnight. In the morning, the medium was replaced with PIPES buffer (25 mM PIPES, pH 7.0, 1 mM $CaCl_2$, 100 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 5.6 mM glucose, 0.1 % BSA), and secretion

was triggered by exposure to 20 μg/ml DNP-BSA for 30 min. The β-glucuronidase activity of cell supernatants and 5% Triton X-100 cell lysates was measured spectrophotometrically at 540 nm as the hydrolysis of phenolphthalein glucuronic acid by the method of Talalay et al., J. Biol. Chem., Vol. 166, pp. 757-772 (1946).

Rat peritoneal mast cells were obtained from adult Sprague-Dawley rats that were anaesthetized with ketamine 42.8 mg/ml/xylazine 8.6 mg/ml/acepromazine 1.4mg/ml (3 ml/kg), decapitated, and exsanguinated. The peritoneal cavity was then opened with a midline incision and lavaged with iced HEPES-Tyrode's buffer with heparin (HTH) (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.4, 0.1% BSA, 5.6 mM glucose, 30 U/ml heparin). Pooled cells from 6 - 10 rats were pelleted by centrifugation at 400 *g* for 15 min, resuspended in 10 ml HTH, then layered over 30% (20 ml) and 80% (15 ml) Percoll step gradients in HTH and centrifuged at 600 *g* for 18 min at 15°C. The cellular pellet was resuspended in HTH and the Percoll gradient centrifugation repeated. The pellet of the second centrifugation was resuspended in 50 ml PBS-1.2% sucrose and centrifuged at 400 *g*, then resuspended in PBS.

EXAMPLE 2

RNA purification

RNA was harvested from RBL cells detached with trypsin-EDTA and collected by centrifugation at 400 *g* for 5 min, and from brains and lungs rapidly excised from 150-200 g Sprague-Dawley rats killed by CO_2 suffocation and exsanguination. Total cellular RNA was isolated on a guanidine thiocyanate/CsCl gradient, extracted twice with phenol/chloroform, then ethanol precipitated. RNA was dissolved in 0.1% diethyl pyrocarbonate-treated water, quantified by measuring absorbance at 260 nm, evaluated for degradation by agarose-

formaldehyde gel electrophoresis, then frozen until used. mRNA was isolated from total RNA either by oligo-dT cellulose chromatography or magnetic beads.

EXAMPLE 3

PCR amplification of Rab3 isoform cDNA fragments

Two μg of RBL cell poly(A)$^+$ RNA was reverse transcribed by 125 U of M-MuLV reverse transcriptase in a 50 μl reaction containing 2.5 μg each of $(dT)_{18}$ and random octamers, 1mM of each dNTP, and 40 U of RNAsin at 37°C for 30 min, 42°C for 30 min, and 50°C for 15 min. The 5' primer was GCACAGTGGGCATCGACTTCAAGGT, and the 3' primer CAGACCTTTGAGCGCTTGGTGGAT. PCR was performed with 1 μl of AmpliTaq in 100 μl reaction buffer supplemented with 1.5 mM $MgCl_2$, 10% (v/v) DMSO, 1 μM of each primer, 50 μM of each dNTP, and 1 μl of the reverse transcription reaction as template. Six cycles of 94°C for 1 min ramping to 49°C in 3 min, 49°C for 1 min, then 72°C for 1 min, were followed by 24 cycles of 94°C for 1 min, 65°C for 1 min, then 72°C for 1 min, with a final extension at 72°C for 6 min. The product was purified by agarose gel electrophoresis, then ligated into the pCR-II vector. DH5α cells were transformed with the ligation mixture and colonies selected for sequencing. A cognate 385 bp fragment of Rab3C was cloned from rat brain poly(A)$^+$ RNA by RT-PCR using the same primers and reaction conditions.

EXAMPLE 4

Northern blot analysis

[^{32}P]-labeled riboprobes were generated by transcription of linearized pCR-II plasmid cDNA's for 2-3 h at 37°C in a 20 μl reaction mixture containing 10 μg DNA, 1 U/μl RNasin, 20 mM DTT, 0.1 mg/ml BSA, 500 μM GTP, ATP, and UTP, 12 μM CTP, 9 μM [α-^{32}P]CTP (400 Ci/mmol), and 20 U T7 RNA polymerase (Promega) in the

manufacturer's buffer. RNAse-free DNAse I was then added for an additional 15 min, and the probe was purified on a Nick Spin column. cRNA hybridization controls were generated in a 50 μl reaction containing 4 μg DNA, 1 U/μl RNasin, 20 mM DTT, 0.1 mg/ml BSA, 500 μM each NTP, 2.8 μM [^{3}H]UTP (35 Ci/mmol), and either 10 U SP6 RNA polymerase or 20 U T7 RNA polymerase in 1X RNA polymerase buffer. Reactions were incubated for 4 h at 40°C for SP6 or 37°C for T7 and then purified on a Nick-Spin column. cRNA and mRNA were fractionated in 0.9% agarose/formaldehyde gels, then blotted onto Nytran+ or Zeta-Probe membranes. RNA was cross-linked to the membranes by UV irradiation and hybridized with riboprobes (1-2 x 10^6 cpm/ml) at 42°C overnight in 50% formamide, 5X Denhardt's solution, 0.15 mg/ml sonicated salmon sperm DNA, 5X SSPE, and 7% SDS. Blots were then washed at room temperature with several changes of 2X SSC/0.05% SDS and at 65°C for 40 min with two changes of 0.1X SSC/).1% SDS. Bound riboprobes were detected by autoradiography.

EXAMPLE 5

Ribonuclease protection assay

The pCR-II vectors containing the PCR-cloned 385 bp fragments of each Rab3 isoform were linearized either with BamHI for synthesis of anti-sense RNA using the T7 promoter or with XbaI to generate sense-RNA using the SP6 promoter. cRNA controls were synthesized in a 50 µl reaction containing 6 µg of DNA, 1U/µl RNAsin, 10 mM DTT, 100 µg/ml BSA, 500 µM each NTP, 2.5 µM [^{3}H]UTP (40 Ci/mmol) and 20 U SP6 RNA polymerase. The reactions were incubated for 4 h at 40°C, treated with 10 U DNase-I for 30 min at 37°C and purified on a Nick Spin column. Riboprobes were transcribed in a 20 µl reaction containing 3 µg DNA, 1U/µl RNAsin, 10 mM DTT, 100 µg/ml BSA, 500 µM each GTP, ATP, UTP, 12.5 µM [α-^{32}P]CTP (800 Ci/mmol) and 20 U T7 RNA polymerase (Promega) in the manufacturer's buffer. After

incubation for 2 h at 37°C, 4 U of DNAse-I was added for 15 min. Full-length transcripts were isolated by gel purification in 5% acrylamide / 8M urea gels. Ribonuclease protection assays were performed using the RPA II kit (Ambion) according to the manufacturer's instructions. Cognate cRNA (0.1 pmol) and yeast RNA were used as positive and negative controls. Each experiment contained 1 pmol riboprobe and was supplemented with yeast RNA to complete a total of 40 μg RNA. Hybridization was carried out overnight at 45°C. Protected probes were electrophoresed through 5% acrylamide / 8M urea gels and visualized by autoradiography.

EXAMPLE 6

Western blot analysis

Brains, lungs, and pancreas were excised from freshly sacrificed rats, and placed in a five-fold (w/v) excess of ice-cold lysis buffer (10 mM TRIS-HCl, pH 7.4, 10 mM NaCl, 5 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride). Tissue was finely ground with an Ultra-Turrax T25 rotor/stator homogenizer using successive three second bursts at 13,500 rpm, 20,000 rpm, and 24,000 rpm, then quickly frozen in liquid nitrogen and stored at -80°C. RBL cells were lysed by sonication for three 15 sec pulses in lysis buffer. The protein mass of cell and tissue homogenates was measured by Coomassie Blue binding in the manner of Bradford, Anal. Biochem., Vol. 72, pp. 248-254 (1976). Human Rab5A and Rab3A proteins were expressed in *E.coli* and purified as described in Hoffenberg et al., J. Biol. Chem., Vol. 10, pp. 5048-5056 (1995). Rat Rab3A, human Rab3B, and mouse Rab3D cDNA's were subcloned into the pGEX-2T vector, expressed as GST-fusion proteins in the DH5a strain of *E.coli* and purified on glutathione Sepharose 4B columns. The mass of GST-Rab3 fusion proteins was determined by laser densitometry of Coomassie-stained acrylamide gels using BSA as a standard. Proteins were electrophoresed through 12%

acrylamide gels, then electrotransferred at 30 V overnight to Immobilon-P membranes. Western blots were blocked with a solution of 5% BSA in TBST (0.05% Tween-20, 10 mM TRIS-HCl, pH 8, 150 mM NaCl), then incubated with primary antibodies in blocking buffer for 1 h at room temperature. Pan-Rab3 monoclonal antibody 42.1 raised against Rab3A was used at 1:5,000 dilution (Fig. 1); affinity-purified antipeptide antibody against the N-terminus of Rab3A was used at 1:5,000; affinity purified antipeptide antibody against the C-terminus of Rab3A was used at 1:10,000; pan-Rab3 polyclonal antiserum raised against Rab3B was used at 1:500. This last antibody was also preadsorbed by diluting 30 μl serum with PBS to 100 μl and incubating it with 50 μl each of glutathione-Sepharose 4B saturated with GST-Rab3A and GST-Rab3D for 12 h at 4°C. The supernatant of a 5 min, 12,000 g centrifugation was then used at 1:10,000. Blots with primary antibody bound were washed six times with TBST and developed with a chemiluminescence kit using horseradish peroxidase-conjugated secondary antibodies according to the manufacturer's instructions (Amersham).

EXAMPLE 7

Immmunocytochemistry

RBL cells were grown on glass coverslips for eight days in the presence of 100 μM quercetin to increase granule expression by the method of Trnovsky et al., Biochem. Pharmacol., Vol. 46, pp. 2315-2326 (1993). Peritoneal mast cells were air-dried on poly-L-lysine-coated coverslips. Cells were then fixed, permeabilized, and incubated with primary antibodies as described in Moore et al., J. Cell Sci., Vol. 108, pp. 2983-2991 (1995). Monoclonal antibodies against AD1 and RMCP II (157/94, Moredun, United Kingdom) were diluted 1:200; polyclonal anti-Rab3B and anti-Rab3D antibodies were diluted 1:50. After three washes in PBS, secondary antibodies (Molecular Probes) were diluted

1:200 in the same buffer as the primary antibodies and incubated with the specimens in darkness for 1 h. The coverslips were again washed, then mounted with Mowiol containing 10% 1,4-diazobicyclo-[2.2.2]-octane, and allowed to dry overnight in darkness. Epifluorescent images were acquired using a Nikon Optiphot 2 microscope with a photographic camera or a Zeiss Axiophot with a Hamamatsu C5810 CCD camera. To detect simultaneously the distribution of two different markers, stained cells were analyzed in a Molecular Dynamics Multiprobe 2001 CLS confocal imaging system attached to a Zeiss Axiovert 100 microscope.

EXAMPLE 8

Assessment of the secretory phenotype of RBL cells

RBL cells are transformed rat mucosal mast cells that have been used as a source of RNA to clone cDNA's of several mast cell-specific proteins. They are a functional model in that they rapidly secrete the contents of their preformed granules in response to cross-linking their surface IgE receptors. The secretory activity of RBL cells declined with time in culture, generally becoming insubstantial by ten cell passages. Since the molecular defect(s) responsible for the loss of secretory phenotype was not known, antigen-stimulated β-glucuronidase release was periodically assessed to insure that Rab3 expression was evaluated in secretion-competent cells. Low passage number RBL cells stimulated with cross-linked IgE released 50-80% of their β-glucuronidase activity within 30 min. In a representative assay RBL cells were stimulated to secrete the contents of their preformed granules by cross-linking IgE receptors with DNP-specific IgE and antigen (DNP-BSA). β-glucuronidase activity was then measured. The representative assay contained one sample and three controls; Triton X-100 total cell lysate; control culture supernatant of cells exposed to DNP-BSA alone; control culture supernatant of cells exposed to IgE alone; and culture

supernatant of cells exposed to both IgE and DNP-BSA. As a result, 74% of cell-associated β-glucuronidase was secreted (Fig. 1). Cells that failed to release more than 50% of their β-glucuronidase activity upon stimulation were no longer used.

EXAMPLE 9

PCR amplification of Rab3 isoforms

The inferred amino acid sequences of all known mammalian isoforms of Rab3 were aligned with H-Ras (Fig. 2). Accession numbers were listed after the protein's name, conserved G-domains were shaded, the regions corresponding to the Rab3-specific PCR primers used herein were double-underlined, the peptide antigen used for the Rab3D-specific antiserum is shown in outline (Baldini et al., Pro. Natl. Acad. Sci., USA, Vol. 89, pp. 5049-5052 (1995); Valentijn et al., Eur. J. Cell Biol., Vol. 70, pp. 33-41 (1996)), and the mapped epitope of pan-Rab3 monoclonal antibody 42.1 was underlined by the method of Baumert et al., Biochem. J., Vol. 293, pp. 157-163 (1993). The rat Rab3B sequence is a composite of a previously cloned fragment (Oberhauser et al., FEBS Lett., Vol. 339, pp. 171-174 (1994)) and the PCR product reported in this manuscript. The rat Rab3D sequence was originally reported as Rab16 (Elferink et al., J. Biol. Chem., Vol. 267, pp. 5766-5775 (1992)), but is 98% identical to mouse Rab3D when corrected for two sequencing errors that resulted in a local frame shift encompassing 12 amino acids (a missed guanine in the first position of the codon for alanine 97 and an extra cytosine in the codon for glutamine 110).

RT-PCR was performed to generate probes of known or novel mast cell Rab3 isoforms for use in mRNA expression studies. PCR primers were designed that averaged 87% identity with the four known rat Rab3 cDNA's (Rab3A - 92% and 87%, 3B - 88% and unknown, 3C - 84% and 83%, 3D - 96% and 79%), but only about 40% identity with

other Rabs. The 5' primer overlaid the "effector" domain, and the 3' primer was in the Rab3-specific but isoform-non-specific portion of the variable C-terminus (Fig. 2). PCR amplification of RBL cell reverse transcripts yielded the predicted 385 bp product (not shown). A restriction digest of the product with the frequent cutter HinfI produced multiple fragments which did not sum to 385 bp (not shown), suggesting that more than one molecular species was present. The PCR product was subcloned and the inserts of twenty-seven colonies sequenced. Of these, 9 were identical to a 385 base fragment of rat Rab3A, 17 were identical to a 385 base fragment of rat Rab16 (homolog of mouse Rab3D - see Fig. 2), and one was 98% identical at the amino acid level to human Rab3B and overlapped a recently cloned fragment of rat Rab3B (Fig. 2). PCR amplification of rat brain reverse transcripts to obtain a Rab3C probe yielded 385 bp Rab3A and Rab3C products (not shown).

EXAMPLE 10

Northern blot analysis

To estimate the relative levels of expression of Rab3 isoforms in RBL cells, Northern blotting was performed using riboprobes transcribed from the PCR products. The relative efficiency of hybridization of the riboprobes to cognate and non-cognate Rab3 isoforms was assessed using serial dilutions of blotted cRNA's of all four Rab3 isoforms. This varied from no apparent distinction between cognate and non-cognate isoforms on some occasions to approximately three-fold more efficient hybridization to cognate cRNA on other occasions (not shown). A Northern blot was performed in which a 385 nucleotide Rab3B coding region riboprobe was hybridized with 4 μg each of poly(A)$^+$ RNA from rat brain, rat lung, and RBL cells. The Northern blot using the Rab3B probe revealed three Rab3 transcripts in RBL cells approximately 3.1, 2.0, and 1.1 kb in length (Fig. 3). Two

of these correspond in size to transcripts described previously in rat tissues: 1.1 kb for Rab3B in pituitary (Lledo et al., Nature, Vol. 364, pp. 540-544 (1993)), and 2.0 kb for Rab3D in brain (Elferink et al., J. Biol. Chem., Vol. 267, pp. 5766-5775 (1992)). Lung mRNA was included as a control because it is known to be enriched with Rab3D transcripts (Baldini et al., Proc. Natl. Acad. Sci., USA, Vol. 89, 5049-5052 (1992); Elferink et al., J. Biol. Chem., Vol. 267, pp. 5766-5775 (1992)), and it showed a strong band at 2.0 kb. Additional Rab3D transcripts of 2.6 (*Id.*), 3.2 and 4.0 kb (Regazzi et al., J. Cell Sci., Vol. 109, pp. 2265-2273 (1996)) have also been described, of which the 3.2 kb transcript likely corresponds to the 3.1 kb transcript detected in RBL cells (Fig. 3). There was no hybridization to RBL cell mRNA at 1.4 kb, the size of rat brain Rab3A (Lledo et al., Nature, Vol. 364, 540-544 (1993); Elferink et al., J. Biol. Chem., Vol. 267, pp. 5766-5775 (1992)), even though there was a strong band in control brain mRNA (Fig. 3). There was no hybridization to either RBL cell or tissue mRNA at 9.5 kb, the size of rat Rab3C (Viggeswarapu and Wildey, Biochem. Biophys. Res. Commun., Vol. 227, pp. 645-650 (1996)). Similar results for the RBL cell 2.0 and 3.1 kb Rab3 transcripts were obtained using Rab3A and Rab3C riboprobes, but there was no visible band at 1.1 kb as there was with the Rab3B riboprobe (not shown). Together, these results suggested that Rab3D transcripts of 2.0 and 3.1 kb are relatively abundant in RBL cells, Rab3B transcripts of 1.1 kb are considerably less abundant, and Rab3A and 3C transcript are not present.

EXAMPLE 11

RNase protection assay

In preliminary studies, it was found that cRNA's of each Rab3 isoform protected their cognate probes in a concentration-dependent fashion and failed to protect non-cognate probes (not shown). Using up to 40 μg total RNA from RBL cells, only the Rab3D probe was

protected, but its autoradiographic band was faint (not shown). Poly(A)$^+$ RNA was then used to increase the sensitivity of the assay. An autoradiogram of the products of an RNase protection assay following polyacrylamide gel electrophoresis was performed. The first four lanes contained 8 μg each of poly(A)$^+$ RNA, the fifth lane contained 4 μg and the sixth lane contained 1.6 μg. A signal was evident with the Rab3D probe using only 1.6 μg of poly(A)$^+$ RNA when no signal was apparent using 8 μg of poly(A)$^+$ RNA with the other probes (Fig. 4). A representative assay that was performed four times is shown. In overexposed autoradiograms, the Rab3D probe was seen to be protected by as little as 0.8 μg of poly(A)$^+$ RNA (not shown), suggesting that Rab3D transcripts are at least 10-fold more abundant in RBL cells than transcripts of other Rab3 isoforms.

EXAMPLE 12

Western blot analysis

Monoclonal antibody 42.1 was raised against Rab3A, but is known to recognize Rab3B and Rab3C as well (Baumert et al., Biochem. J., Vol. 293, pp. 157-163 (1993)). Monoclonal antibody 42.1 was used to probe Western-blotted proteins. The first lane contained 2.5 ng purified Rab3A, the second lane contained 30 μg pancreatic homogenate, the third lane contained 5 ng purified Rab5A, the fourth lane contained 3 μg brain homogenate, the fifth lane contained 100 μg RBL cell lysate and the sixth lane contained 3 μg Rab3D-expressing *E. coli* lysate. This antibody yielded greater immunoreactivity against 3 μg of rat brain than against 100 μg of RBL cell lysate or 30 μg of pancreas, a tissue known to express Rab3D (Ohnishi et al., Am. J. Physiol. Gastrointest. Liver Physiol., Vol. 271, pp. G531-G538 (1996); Valentijn et al., Eur. J. Cell. Biol., Vol. 70, pp. 33-41 (1996)) (Fig. 5). Immunoreactivity to rat peritoneal mast cells was similar to that of RBL cells (not shown). To determine whether the ~100-fold greater Rab3 immunoreactivity in

brain than RBL cells and mast cells accurately reflected total relative Rab3 protein abundance, the relative immunoreactivity of antibody 42.1 toward Rab3 GST-fusion proteins was assessed. The antibody was ~10-fold more reactive toward Rab3A than toward Rab3B or 3D (not shown). Since Rab3D is the major Rab3 isoform in RBL cells and mast cells (see below) while Rab3A is the major isoform in brain (Sudhof, Nature, Vol. 375, 645-653 (1995)), this result suggested that total Rab3 proteins are ~10-fold more concentrated in brain than in mast cells.

Affinity purified Rab3D antiserum was used to probe Western-blotted proteins. The first lane contained 5 ng purified Rab3A, the second lane contained 15 μg pancreatic homogenate, the third lane contained 5 ng purified Rab5A, the fourth lane contained 100 μg brain homogenate, the fifth lane contained 100 μg RBL cell lysate and the sixth lane contained 15 μg Rab3D-expressing *E. coli* lysate. The Rab3D-specific antibodies reacted with a 24 kDa band in RBL lysate present in greater abundance than in brain but less than in pancreas (Fig. 6).

Twenty-five μg of RBL cell lysate and increasing amounts of GST-Rab3D were compared by immunoblot analysis using Rab3D-specific antiserum. The mass of GST-Rab3D was expressed as ng Rab3D protein. The resulting immunoblot was densitometrically scanned and plotted. The titration of Rab3D immunoreactivity in RBL lysate against dilutions of GST-Rab3D yielded a concentration of 30 pg Rab3D per μg RBL lysate (28 and 32 pg/μg in two separate experiments) (Fig. 7). In a test of the specificity of the Rab3D antibodies, they reacted with Western-blotted GST-Rab3D but failed to reacted with 100-fold more GST-Rab3A and GST-Rab3B (not shown). A polyclonal antiserum raised against Rab3B reacted with RBL lysate, but was also found to recognize GST-Rab3D and GST-Rab3A, albeit

with ~10-fold lower reactivity than GST-Rab3B (not shown). After adsorbing out Rab3A and Rab3D antibodies, the antiserum recognized GST-Rab3D with ~300-fold lower reactivity than GST-Rab3B and failed to generate immunoreactivity greater than 0.5 pg Rab3B per μg lysate (not shown). Below this level, it was not possible to determine whether the weak reactivity was due to Rab3B, cross-reactivity with Rab3D, or non-specific interaction with an unrelated protein. Rab3D was found to be at least 60-fold more abundant than Rab3B in RBL cells. Rab3A-specific antiserum similarly failed to react with RBL cell lysate (not shown), and Rab3C-specific antiserum was not tested because no Rab3C transcripts were detected by RT-PCR, Northern blot, or RNase protection assay (see above).

EXAMPLE 13

Immunofluorescent localization of Rab3D and secretory granules

Cells were imaged by indirect immunofluorescence using primary antibodies specific for the proteins indicated and FITC-conjugated secondary antibodies. The proteins screened were: AD1; RMCP-II; pan-Rab3 (polyclonal antiserum); and Rab3D.

RBL cell secretory granules were visualized using monoclonal antibodies specific for the granule membrane protein AD1 (Fig. 8A) and the secretory protease RMCP-II (Fig. 8B). Immunofluorescence microscopy demonstrated punctate structures in elongated cytoplasmic processes and in the perinuclear region. The largest granules were approximately 1μm in diameter, typical of the size of mature mast cell granules, but most were substantially smaller than this. Control antibodies, including non-immune primary sera, irrelevant primary antibodies, or secondary antibodies alone, all failed to generate punctate immunofluorescence (not shown). The morphology of the RBL cells, ranging from polygonal or rounded, to elongated with lengthy dendritic

processes, varied with cell density and time in culture. Inclusion of quercetin in the culture medium substantially increased the number of cells containing immunocytochemically apparent granules and increased the number of granules per cell.

No punctate immunofluorescence was observed using the pan-Rab3 monoclonal antibody 42.1 (not shown). The polyclonal antiserum raised against GST-Rab3B that also reacts with other Rab3 isoforms yielded a punctate immunofluorescence pattern similar to that of AD1 and RMCP-II (Fig. 8C). The same antiserum after preadsorption yielded no punctate immunofluorescence and was indistinguishable from negative controls (not shown). Antibody specific for Rab3D showed punctate immunofluorescence similar in distribution to that of AD1, RMCP-II, and the pan-Rab3 polyclonal antiserum (Fig. 8D).

Cells were labeled with antibodies to RMCP-II or Rab3D, then imaged by indirect immunofluorescence laser confocal microscopy using Texas Red and FITC labeled secondary antibodies. In the computer-merged images, yellow color indicated coincident red and green fluorescence. Arrows pointed to punctate structures visualized with both fluorochromes, arrowheads pointed to punctate structures visualized with only one fluorochrome.

Scanning laser confocal microscopy used to examine the colocalization of Rab3D with the secretory granule marker RMCP-II showed a similar size range of punctate structures with both antibodies (Fig. 9). The spatial distribution of each antibody was also similar, with most of the perinuclear punctate structures aggregated on the same side of the nucleus. Occasional granules were found in the cell periphery. The density of granules makes interpretation of the merged images difficult, but by switching between the red and green channels

on the computer screen, it was clear that some granules were stained with both primary antibodies (*e.g.* arrows, Fig. 9), but that most stained with only one antibody (*e.g.* arrowheads, Fig. 9).

Mature mast cells obtained by peritoneal lavage and gradient centrifugation were more than 95% pure as judged by phase contrast microscopy showing abundant large, refractile cytoplasmic granules in freshly isolated cells (not shown). This purity was confirmed by Alcian blue staining of the secretory granules (not shown). Immunocytochemistry revealed extensive colocalization of punctate Rab3D staining with the refractile cytoplasmic granules (Fig. 10). The isoform non-specific polyclonal antiserum raised against GST-Rab3B yielded the same result, but the preadsorbed Rab3B antibodies showed no specific immunofluorescence (not shown). Rat peritoneal mast cells were allowed to air dry on glass slides, then labeled with antibodies to Rab3D and imaged by phase contrast microscopy or by indirect immunofluorescent microscopy using Texas Red-conjugated secondary antibodies. RBL cells cultured on coverslips were activated for secretion by cross-linking bound IgE with specific antigen. After 15 min they were fixed, labeled with antibodies to Rab3D, then imaged using Texas Red-conjugated secondary antibodies. Approximately one third of the fixed cells were substantially smaller than the highly granulated cells and showed Rab3D immunoreactivity in a linear distribution at the cell periphery (Fig. 10 A-D). These smaller cells are mast cells which partially degranulated during fixation, resulting in a reduction in cellular volume and translocation of Rab3D to the plasma membrane. To confirm that Rab3D translocates upon exocytosis, peritoneal mast cells were allowed to bind IgE overnight, then triggered with specific antigen. Fifteen min after the addition of antigen, virtually all of the cells displayed linear peripheral Rab3D staining (not shown). A similar

result was obtained upon triggering exocytosis of RBL cells (Fig. 10 E-F).

EXAMPLE 14

Synaptotagmin2 expression in RBL cells

Based upon evidence of the structural homology of the neuronal and mast cell machines, homologs of other components of the neuronal machine were sought using the same strategy. Since mast cell exocytosis is well-known to be calcium dependent and synaptotagmins appear to be exocytic calcium sensors, it is desirable to analyze the expression of synaptotagmins in RBL cells. The nine known mammalian Syt isoforms share a high degree of sequence identity in their C_2 calcium binding domains, facilitating an RT-PCR strategy for cloning all expressed Syt isoforms. Nested PCR reactions yielded a product of the predicted size, 365 bp (Fig. 12). This product was cloned using the vector pCRII (Invitrogen), and isoforms 2, 3, and 5 were identified by sequencing 21 subclones (Fig. 13). This result was consistent with a restrictase analysis of the initial PCR product, suggesting that the subcloning analysis fairly represented the amplification of Syt isoforms by PCR. Since RT-PCR cloning is known not to quantitatively reflect mRNA expression unless specifically controlled for this purpose, quantitative RNase protection assays were performed using probes transcribed from the PCR subclones to assess relative expression of the three cloned Syt isoforms. A representative assay that was performed three times in which 4 μg of mRNA were hybridized with ^{32}P-labeled riboprobes of Syt1, included as a negative control, and Syt2, 3 and 5. Following polyacrylamide gel electrophoresis, an autoradiogram was developed (Fig. 14). The Syt2 probe was best protected by RBL cell mRNA, indicating that it is the major Syt isoform in RBL cells.

EXAMPLE 15

Sec1/Munc18 Expression in RBL Cells

Using an approach similar to that for Rab3 and Synaptotagmin, primers were designed to amplify all known isoforms of Sec1 by PCR from RBL cell reverse transcripts. A product of the expected size was obtained and subcloned. Of 20 colonies subjected to sequencing, 19 were Sec1-B and 1 was Sec1-A. Thus, Sec1-B is the major isoform in RBL cells.

EXAMPLE 16

Mapping the Rab3D Gene in Mice

A genomic clone encoding the Rab3D gene was fluorescently labelled and hybridized to several mouse chromosomes (FISH analysis - flourescent in situ hybridization see Figure 15). The Rab3D gene mapped to 13A2-3, which is syntenic with a human asthma locus (6p23-p21.3).

EXAMPLE 17

Altering Expression of Rab3D in Cells and Whole Animals

The mouse Rab3D gene is ablated by homologous recombination using standard genetic methods. In brief, two genomic clones (4 and 5 in Figure 16) that cover the structural gene were isolated. These were digested to provide long flanking arms for homologous recombination; the strucural gene was replaced by the LacZ reporter gene and neomycin gene; and the thymidine kinase gene was added outside the targeting construct (see "Construct" in Figure 16). The product of homologous recombination is shown on the bottom of Figure 16. Embryonic stem cells are electroporated with this construct, then negatively and positively selected with G418 and FIAU. Clones are screened for homologous recombination by Southern blot analysis, then microinjected into blastocoels to generate chimeric mice. These are

then bred to generate mice homozygous for the Rab3D deletion. Mast cells harvested from the peritoneal cavity of "knockout" are studied by capacitance measurement through a patch pipette. The whole animal is studied in models of asthma, anaphylaxis and peritonitis. Rab3D wild-type and mutant protein is overexpressed in RBL cells using G418 selection and in mice using the FcεRI-α promoter. Cellular and whole animal physiology is studied. In addition to providing a powerful approach to analysis of protein function, this method provides a model of therapeutically disrupting the function of this protein to assess its value. In cases where the homozygous deletant is embryonic lethal, mast cells can be differentiated from embryonic stem cells by addition of IL-3 and SCF (stem cell factor), making this an ideal system for study of secretory function.

EXAMPLE 18

Overexpressing Sec1-B Wild-type and Mutant Protein in Cells and Whole Animals

Sec1-B has been cloned in a eukaryotic expression vector. It is transfected in cultured mast cell lines, and overexpressing clones selected. In Drosophila, Sec1 overexpression leads to a severe defect in neurotransmitter release. By analogy, overexpression of the major mast cell isoform results in a secretory defect in mast cells. In this case, Sec1-B is overexpressed in mast cells using the FcεRI-α promoter. Peritoneal mast cell secretory function is studied as for Rab3D, and whole animals are studied in pathophysiologic models as for Rab3D. In further experiments, the phosphorylation sites of Sec1-B are analyzed. When these are identified, they are mutated. Since activation of protein kinases can rescue the phenotype of Sec1 overexpression in Drossophila, overexpression of Sec1-B phosphorylation-defective mutants result in a more severe mast cell secretion defect than overexpression of wild-type protein. In addition to providing insight

into the function of Sec1-B, this provides a model for gene therapy in which the human gene or mutated forms thereof can be overexpressed in mast cells.

EXAMPLE 19

Expressing Mammalian Secretory Proteins in Lower Organisms for Analysis of Function

Synaptotagmin1, Synaptotagmin2, and Synaptotagmin3 were expressed in Drosophila under control of neural-specific promotors. These are crossed onto Drosophila in which the endogenous gene was deleted. The ability of mammalian Synaptotagmins to rescue the null Drosophila phenotype is analyzed electrophysiologically. This provides information about the properties of mammalian Synaptotagmins as exocytic calcium sensors, and provides a model system for analysis of pharmacologic strategies targeted at Synaptotagmin function.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned as well as those inherent therein. The methods and procedures, molecules, cell lines and specific compounds described herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the invention. Changes herein and other uses will occur to those skilled in

the art which are encompassed within the spirit of the invention and are defined by the scope of the claims.

What is claimed is:

1. A method of identifying molecular components of the mast cell secretory machine comprising the steps of:

quantitatively measuring the gene expression in a mast cell or mast cell line by measuring a protein or mRNA product;

locating the subcellular site of the protein product of gene expression;

determining whether such product is a component of the mast cell secretory machine.

2. The method of claim 1, wherein the quantitative gene expression is determined by an RNAse protection assay.

3. The method of Claim 1, wherein the quantitative gene expression is determined by quantitative immunoblotting.

4. The method of Claim 1, wherein the quantitative gene expression is determined by Northern blot analysis.

5. The method of Claim 1, wherein the quantitative gene expression is determined by a quantitative reverse-transcription polymerase chain reaction (RT-PCR) assay.

6. The method of Claim 1, wherein the subcellular localization step involves immunofluorescence microscopy.

7. A method for identifying compounds exhibiting an agonistic or antagonistic effect on the mast cell secretory machine comprising of:

identifying the product of the mast cell secretory machine by the method of claim 1; and

screening for compounds that exhibit agonistic or antagonistic effects with said product by testing the compound's activity in the mast cell secretory machine.

8. The method of Claim 7, wherein the mast cell constituent is a protein selected from the group consisting of Rab3, Synaptotagmin, Synaptotagmin2, Synaptotagmin3, Syntaxin, Rabphilin, Cellubrevin/VAMP, Sec1/Rop/Munc18, Sec1-B, SNAP-25, Cysteine string protein, Complexin, SCAMP, Sec3, Sec5, Sec6, Sec8, Sec10, Sec 15, Exo70, NSF and α/β/γ-SNAP.

9. The method of Claim 7, wherein the mast cell constituent is a Rab3d protein.

10. The method of Claim 7, wherein the mast cell constituent is Synaptotagmin2.

11. The method of Claim 7, wherein the mast cell constituent is Synaptotagmin3.

12. The method of Claim 7, wherein the screening comprises an assay of protein-protein interactions.

13. The method of Claim 7, wherein the screening comprises an assay measuring the degree of effect on protein-protein interactions.

14. A method of quantifying the effect of a compound identified by Claim 7 on the mast cell secretory machine comprising an assay determining the amount of degranulation of mast cells.

15. A method of gene therapy for altering mast cell secretion comprising the step of transducing bone marrow stem cells with a gene

construct designed to promote expression of exocytic proteins in mast cells.

16. The method of claim 15 wherein, the stem cells are transduced *ex vivo*.

17. The method of claim 15 wherein, the stem cells are transduced *in vivo*.

18. A method of detecting agonists or antagonists to screen compounds for their ability to alter mast cell secretion comprising:

expressing mammalian mast cell exocytic proteins in lower organisms;

adding the the compound to be tested; and

measuring whether the compound increases or decreases secretion of mast cells.

19. The method of claim 18 where the expressing step is selected from the group of expressing the mammalian protein on top of the endogenous proteins of the lower organism and expressing a mammalian protein substituted in deletants of the endogenous gene.

20. The method of claim 18, wherein the lower organism is fruit fly Drosophila melanogaster or the budding yeast Saccharomyces cerevisiae.

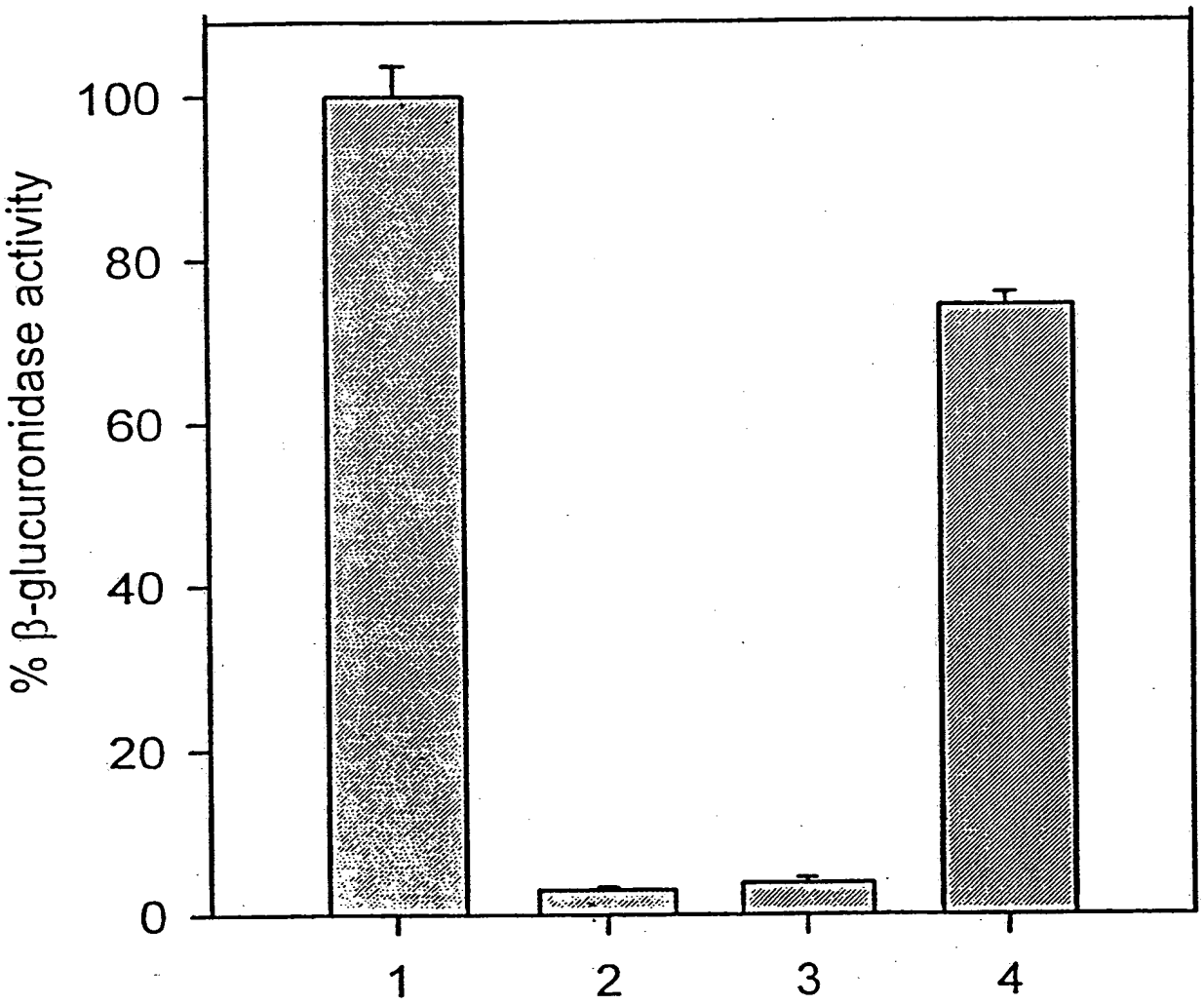


Figure 1

hH-Ras	J00277	MTEY
hRab3A	J04941	MASATDSRYGQKESSDQNFDYMF
rRab3A	X06889	MASATDSRYGQKESSDQNFDYMF
mRab3A	X72966	MASATDSRYGQKESSDQNFDYMF
hRab3B	J04941	MASVTDGKHGVKDASDQNFDYMF
rRab3B	S68807,U68184	...............DQNFDYMF
bRab3C	J03943	MRHEAPMQMASAQDARYGQKDSSDQNFDYMF
rRab3C	D78197	MRHEAPMQMASAQDARFGQKDSSDQNFDYMF
mRab3D	M89777	MASASEPPASPRDAADQNFDYMF
rRab3D	M83681	.....................MF

	5	15	25	35	44
hH-Ras	KLVVVGAGGV	GKSALTIQLI	QNHFVDEYDP	TIEDSYR-KQ	VVIDGETCLL
hRab3A	KILIIGNSSV	GKTSFLFRYA	DDSFTPAFVS	TVGIDFKVKT	IYRNDKRIKL
rRab3A	KILIIGNSSV	GKTSFLFRYA	DDSFTPAFVS	TVGIDFKVKT	IYRNDKRIKL
mRab3A	KILIIGNSSV	GKTSFLFRYA	DDSFTPAFVS	TVGIDFKVKT	IYRNDKRIKL
hRab3B	KLLIIGNSSV	GKTSFLLRYA	DDTFTPAFVS	TVGIDFKVKT	VYRHEKRVKL
rRab3B	KLLIIGNSSV	GKTSFLFRYA	DDTFTPAFVS	TVGIDFKVKT	VYRHEKRVKL
bRab3C	KLLIIGNSSV	GKTSFLFRYA	DDSFTSAFVS	TVGIDFKVKT	VFKNENRIKL
rRab3C	KLLIIGNSSV	GKTSFLFRYA	DDSFTSAFVS	TVGIDFKVKT	VFKNEKRIKL
mRab3D	KLLLIGNSSV	GKTSFLFRYA	DDSFTPAFVS	TVGIDFKVKT	VYRHDKRIKL
rRab3D	KLLLIGNSSV	GKTSFLFRYA	DDSFTPAFVS	TVGIDFKVKT	VYRHDKRIKL

Figure 2

```
                54        64        74        84        94
hH-Ras      DILDTAGQEE YSAMRDQYMR TGEGFLCVFA INNTKSFEDI HQYREQIKRV
hRab3A      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
rRab3A      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
mRab3A      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
hRab3B      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWATQIKT-
rRab3B      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
bRab3C      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
rRab3C      QIWDTAGQER YRTITTAYYR GAMGFILMYD ITNEESFNAV QDWSTQIKT-
mRab3D      QIWDTAGQER YRTITTAYYR GAMGFLLMYD IANQESFTAV QDWATQIKT-
rRab3D      QIWDTAGQER YRTITTAYYR GAMGFLLMYD IANQESFTAV QDWATQIKT-

                104       114       123       133       143
hH-Ras      KDSDDVPMVL VGNKCDLAA- RTVECRQAQD LARSYGIPYI ETSAKTRQGV
hRab3A      YSWDNAQVLL VGNKCDMEDE RVVSSERGRQ LADHLGFEFF EASAKDNINV
rRab3A      YSWDNAQVLL VGNKCDMEDE RVVSSERGRQ LADHLGFEFF EASAKDNINV
mRab3A      YSWDNAQVLL VGNKCDMEDE RVVSSERGRQ LADHLGFEFF EASAKDNINV
hRab3B      YSWDNAQVIL VGNKCDMEEE RVVPTEKGQL LAEQLGFDFF EASAKENISV
rRab3B      YSWDNAQVIL VGNKCDMEEE RVIPTEKGRL LAEQLGFDFF EASAKENISV
bRab3C      YSWDNAQVIL VGNKCDMEDE RVISTERGQH LGEQLGFEFR ETSAKDNINV
rRab3C      YSWDNAQVIL AGNKCDMEDE RVVSTERGQR LGEQLGFEFF ETSAKDNINV
mRab3D      YSWDNAQVIL VGNKCDLEDE RVVPAEDGRR LADDLGFEFF EASAKENINV
rRab3D      YSWDNAQVIL VGNKCDLEDE RVVSAEDGQR LAGDLGFEFF EASAKENINV
```

Figure 2 continued

```
                153       163
hH-Ras      EDAFYTLVRE IRQHDLRKLNPPDESGPGCMSCKCVLS
hRab3A      KQTFERLVDV ICEKMSESLDTADPAVTGAKQGPQLSDQQVPPHQDCAC
rRab3A      KQTFERLVDV ICEKMSESLDTADLAVTGAKQGPQLTDQQAPPHQDCAC
mRab3A      KQTFERLVDV ICEKMSESLDTADPAVTGAKQGPQLTDQQAPPHQDCAC
hRab3B      RQAFERLVDA ICDKMSDSLDT-DPSMLGSSKNTRLSDTPPLLQQNCSC
rRab3B      RQTFERLVD. ..........................
bRab3C      KQTFERLVDI ICDKMSESLET-DPAITAAKQNTRLKETPPPPQPNCGC
rRab3C      KQTFERLVDI ICDKMSESLET-DPAITAAKQSTRLKETPPPPQPNCGC
mRab3D      KQVFERLVDI ICDKMNESLEP-SSSPGSNGKGPALGDTPPPQPSSCSC
rRab3D      KQVFERLVDI ICDKMNESLEP-SSSPGSNGKGPALGDTPPPQPSSCGC
```

Figure 2 continued

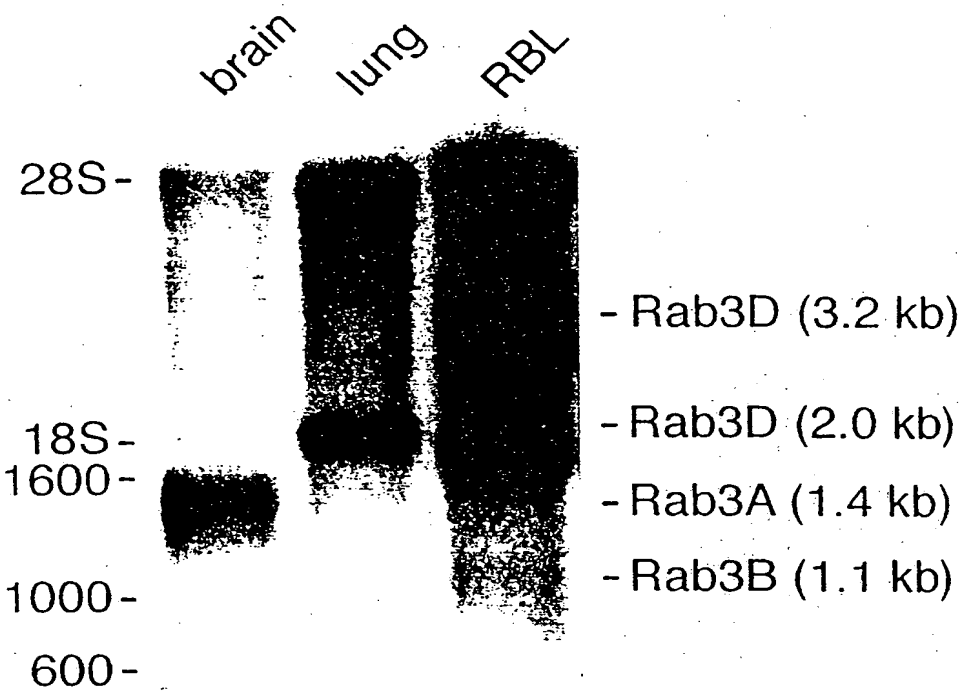


Figure 3

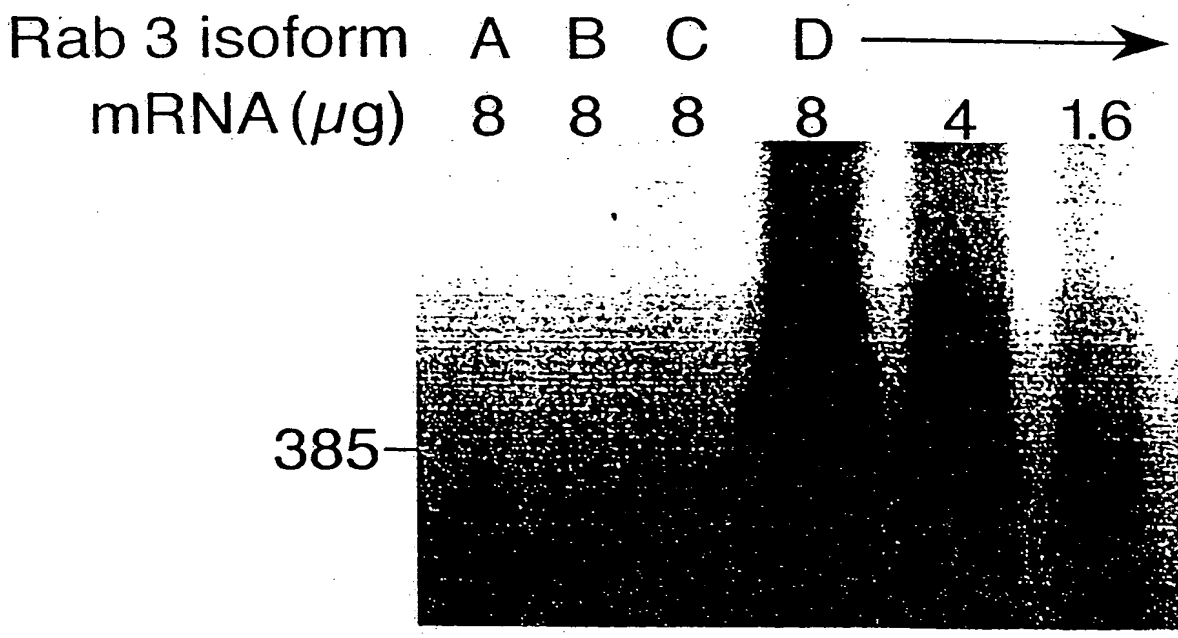


Figure 4

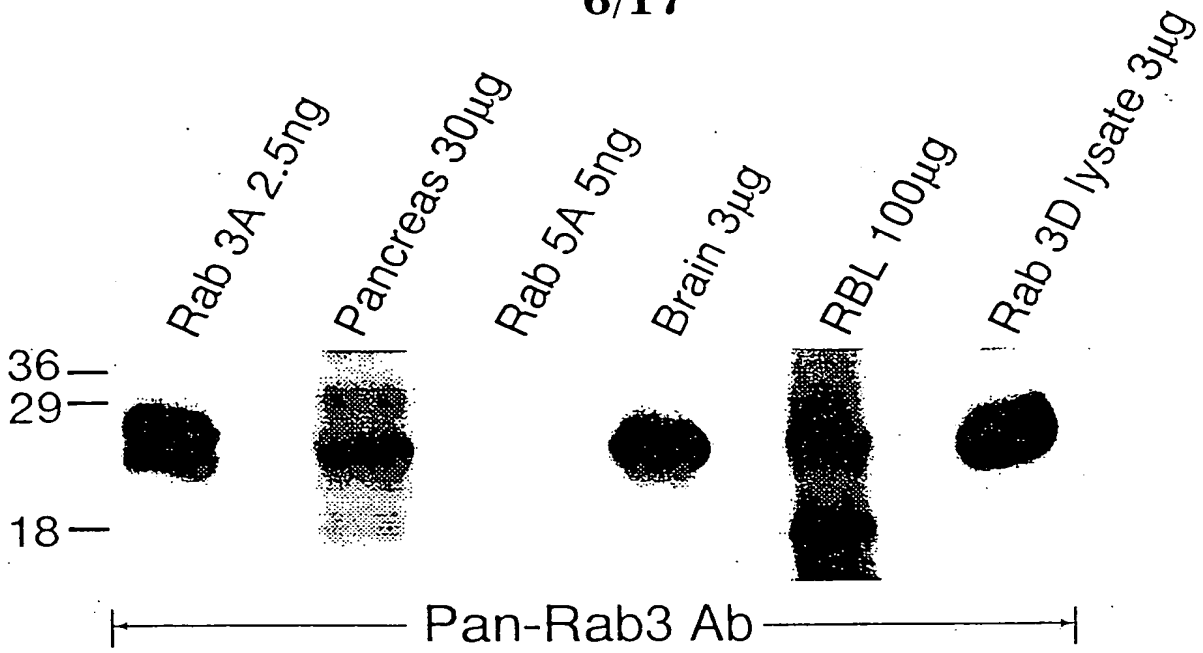


Figure 5

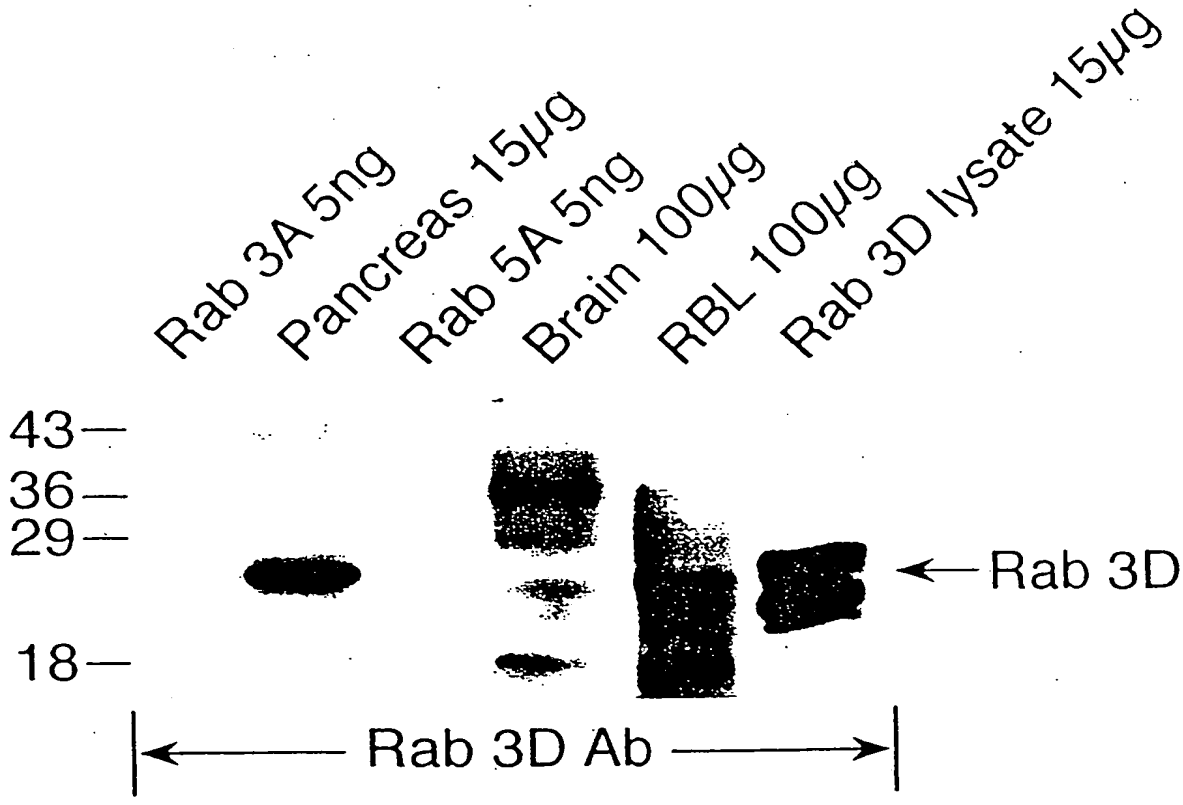


Figure 6

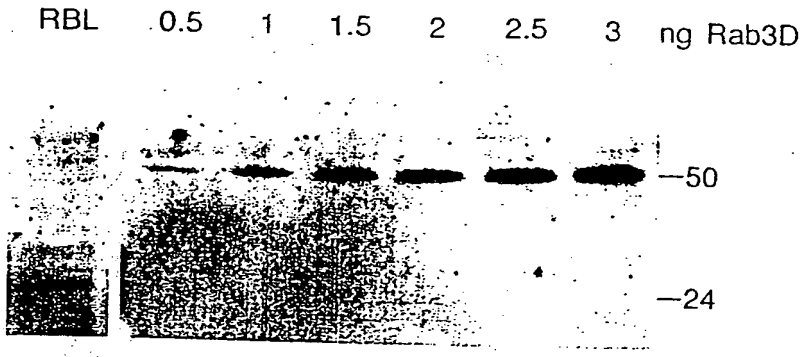


Figure 7A

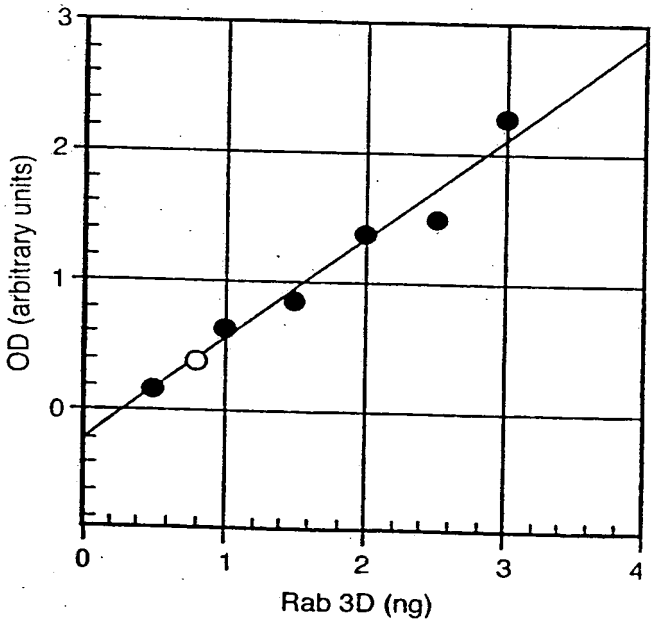


Figure 7B

Figure 8A

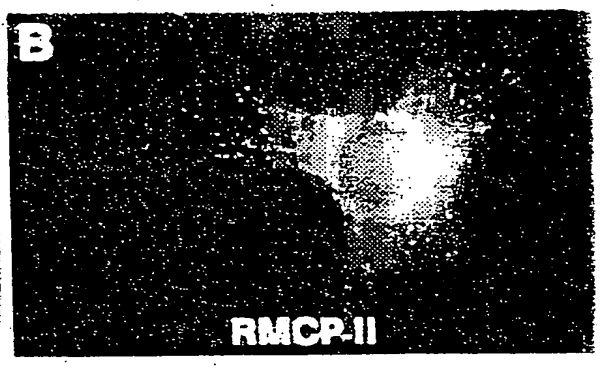


Figure 8B

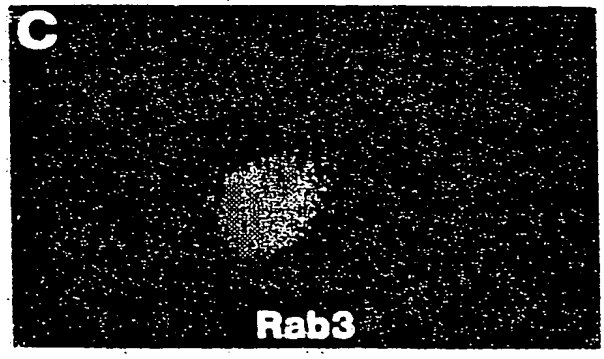


Figure 8C

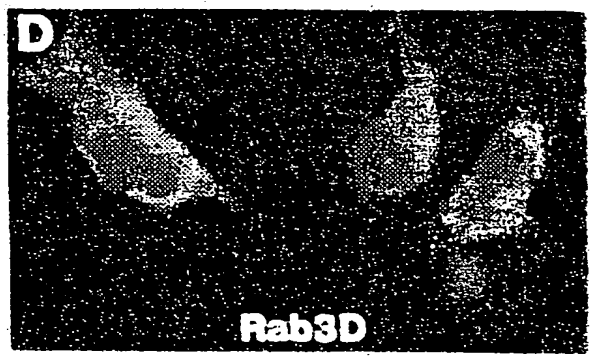


Figure 8D

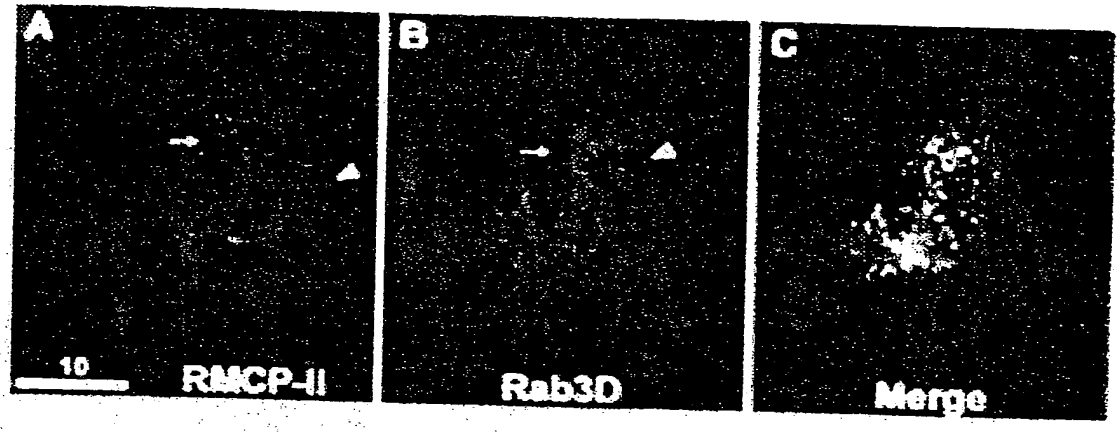


Figure 9A Figure 9B Figure 9C

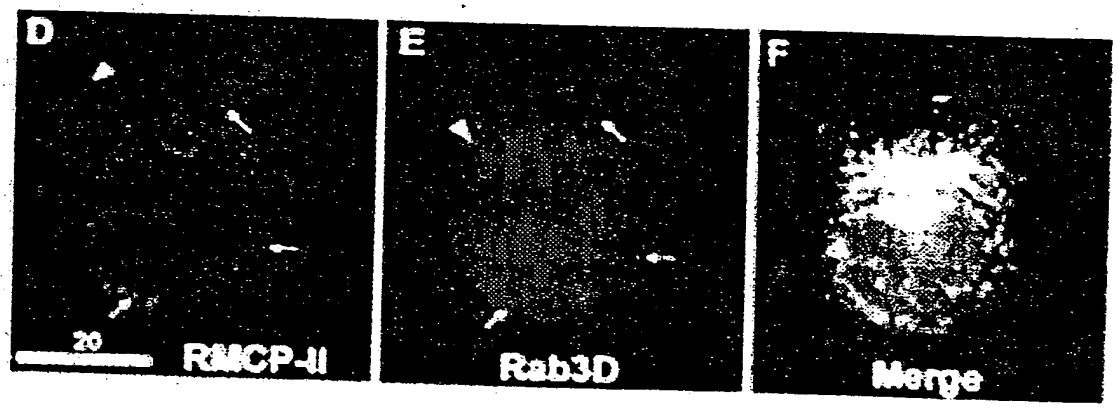


Figure 9D Figure 9E Figure 9F

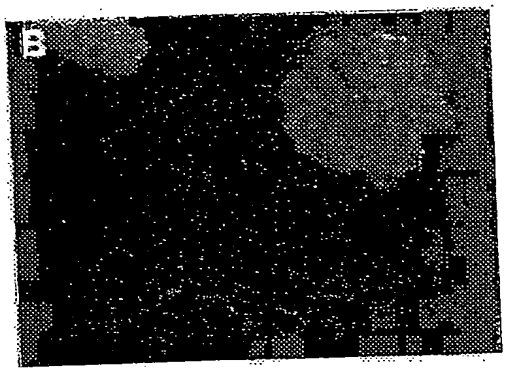


Figure 10A

Figure 10B

Figure 10C

Figure 10D

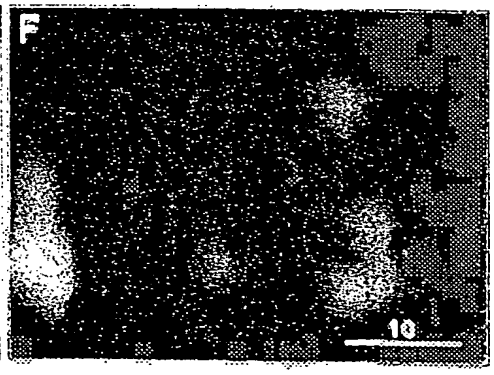


Figure 10E

Figure 10F

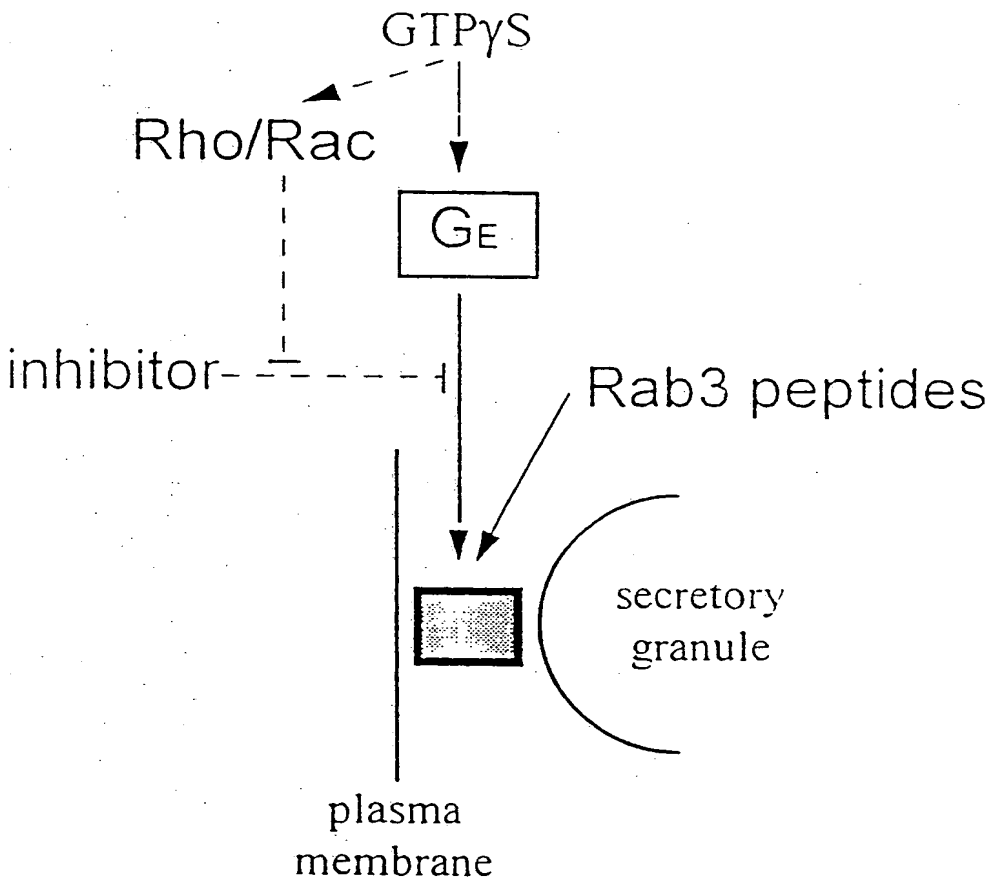


Figure 11

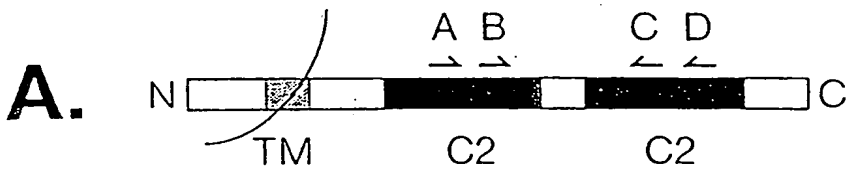


Figure 12A

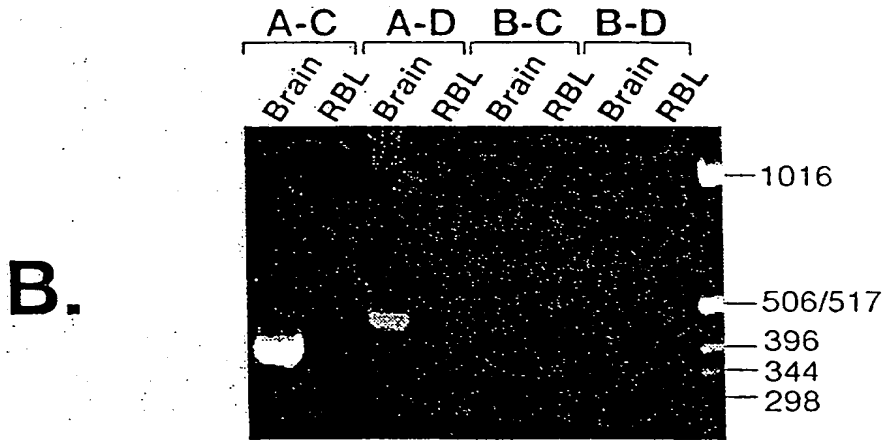


Figure 12B

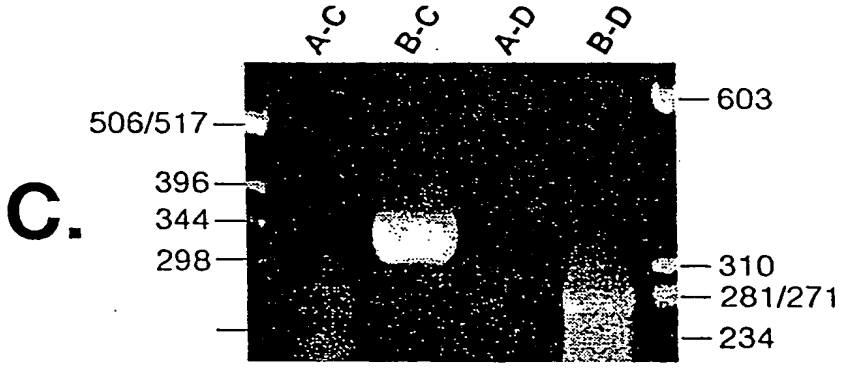
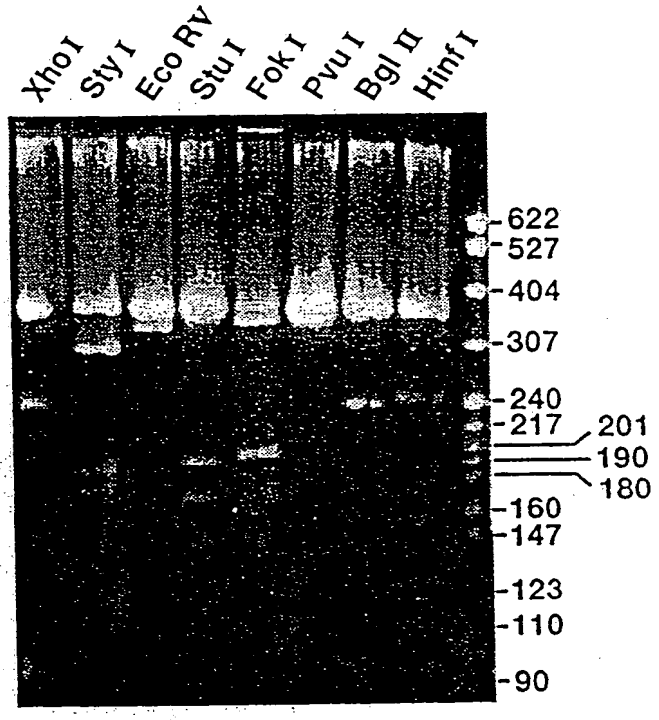


Figure 12C



Syt isoform	1	2	3	4	5	6	7	8	9
Restrictase	?	+	+	-/?	+	-	-	-	-/?
Sequencing	-	10	9	-	2	-	-	-	--

Figure 13

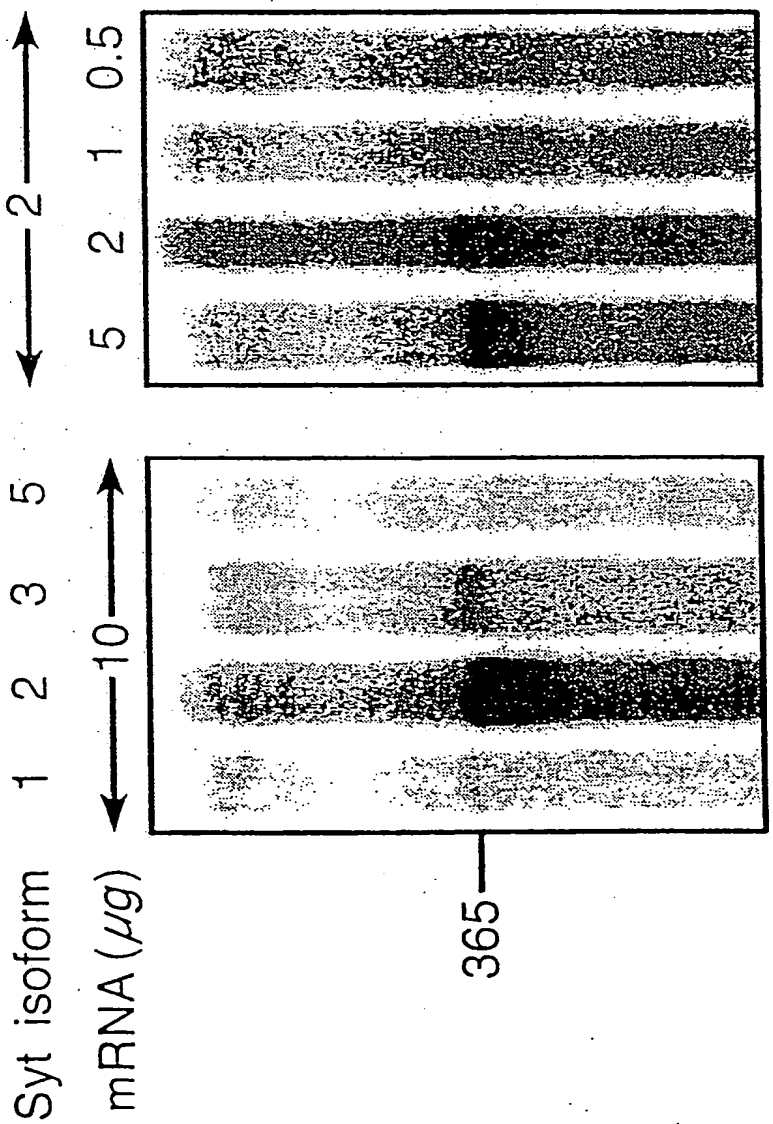


Figure 14

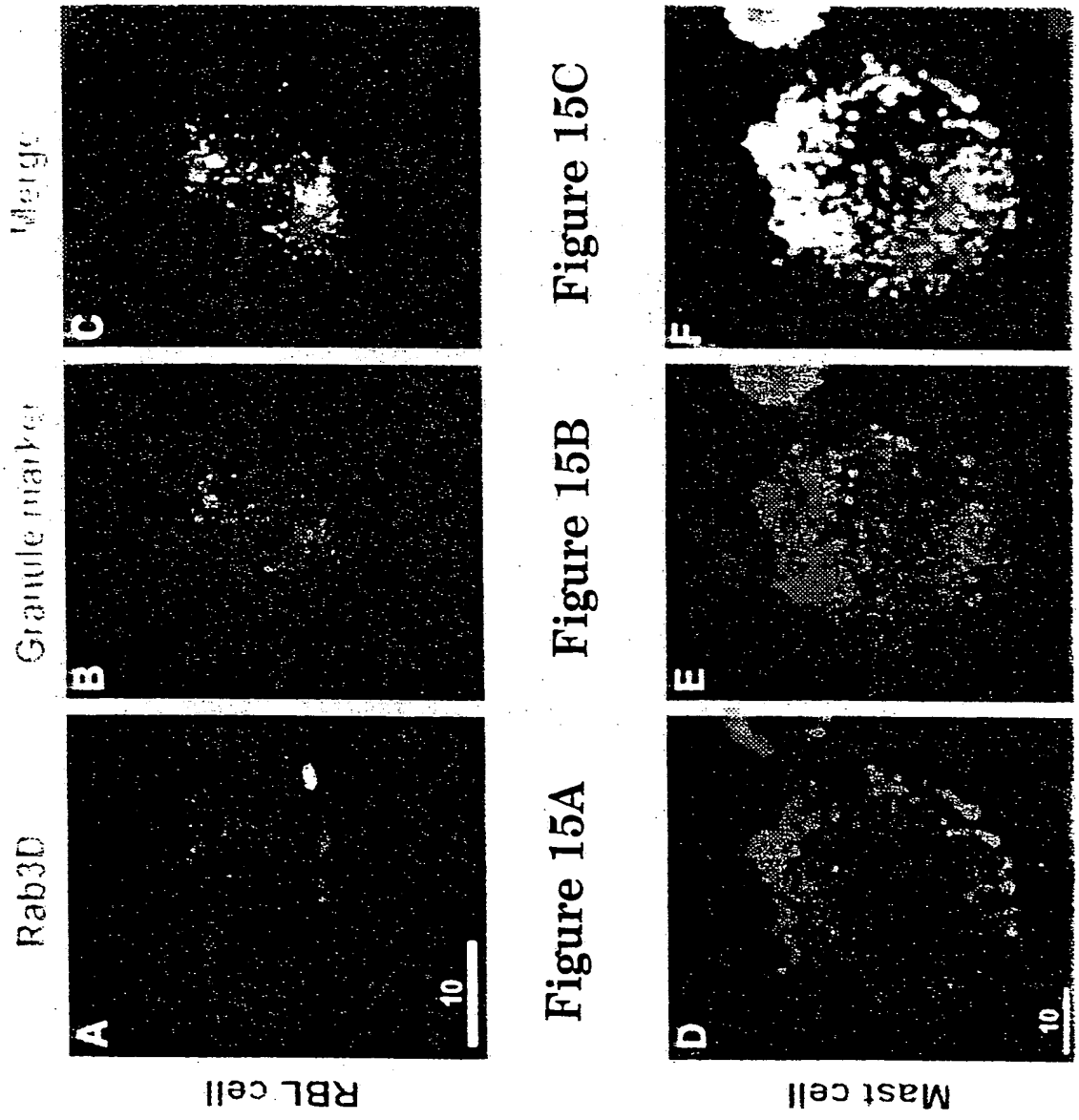


Figure 15A Figure 15B Figure 15C

Figure 15D Figure 15E Figure 15F

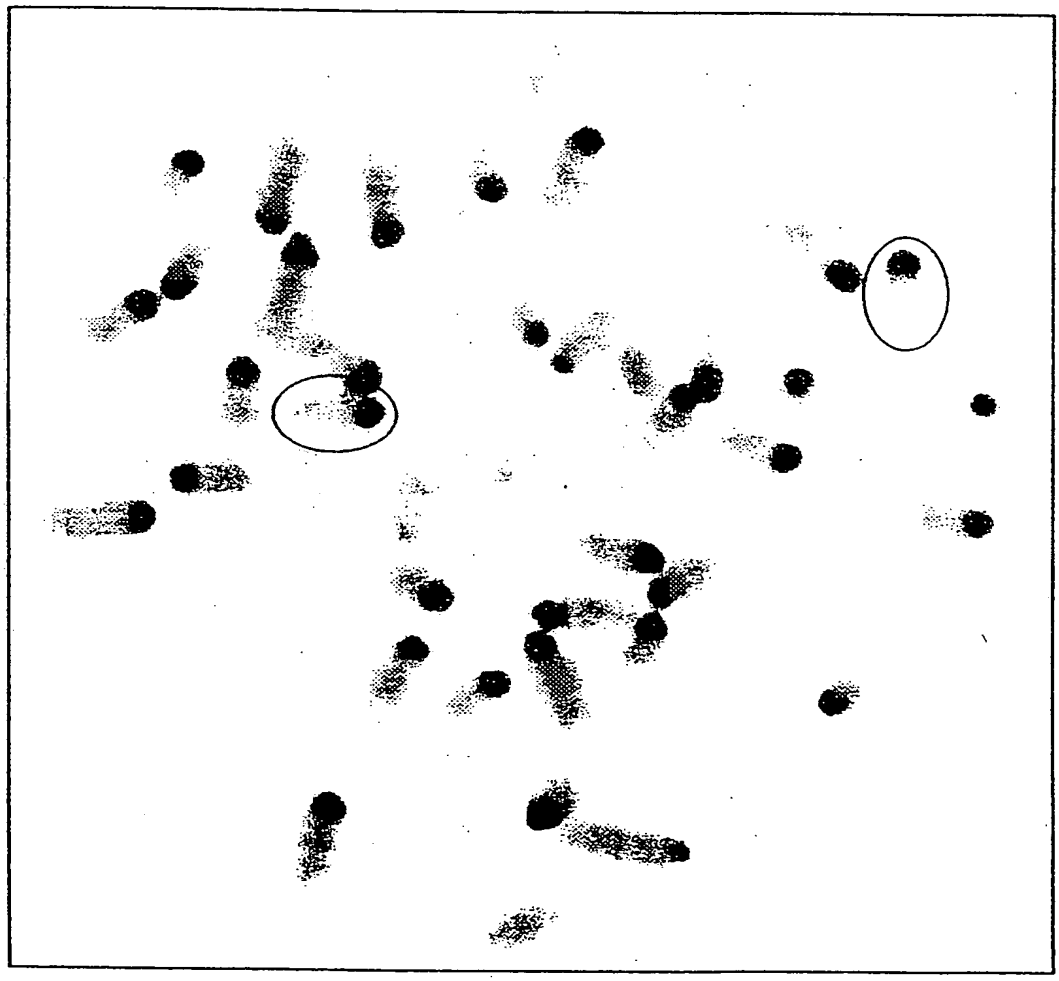


Figure 16

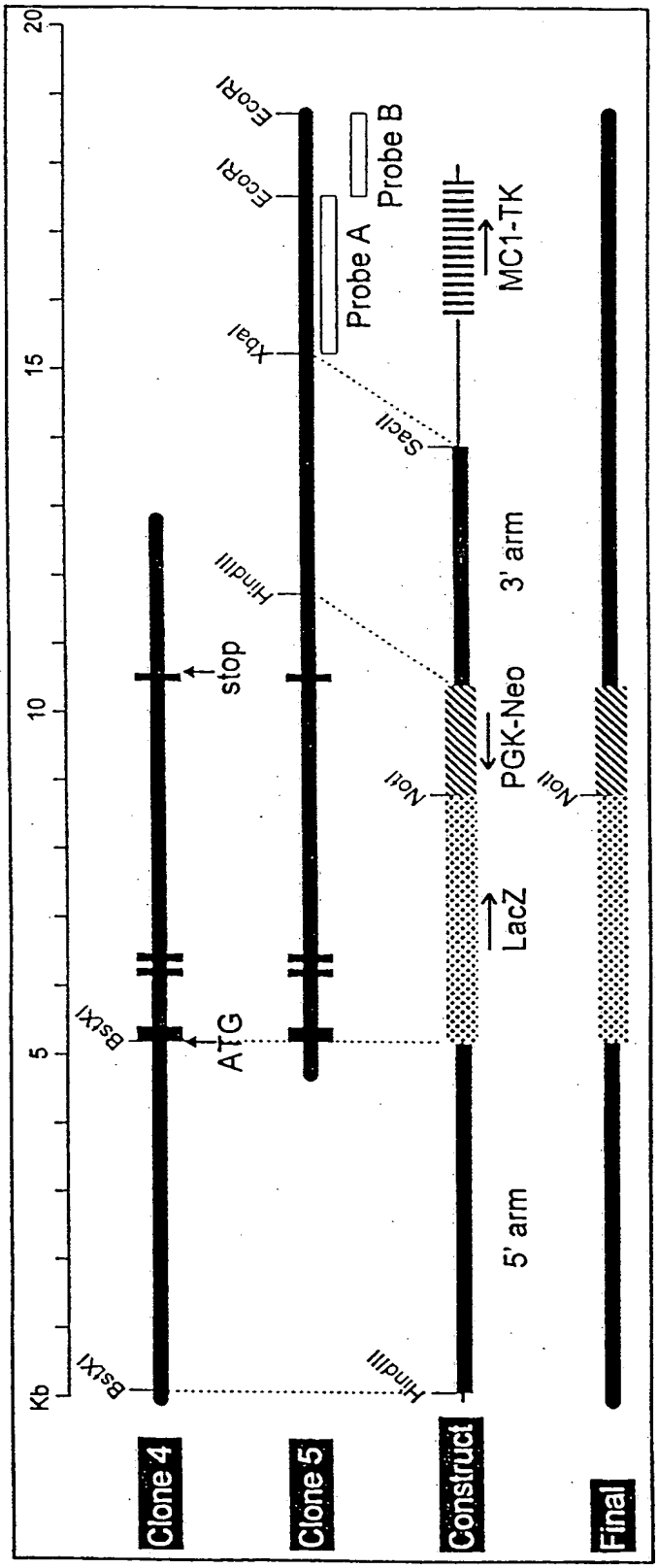


Figure 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11702

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :A61K 48/00; G01N 33/53
US CL :514/44; 435/7.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/7.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

medline biosis caplus wpids aps

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	OBERHAUSER, A.F. RT-PCR cloning of Tab3 isoforms expressed in peritoneal mast cells. FEBS Letters. 1994, Vol. 339, Nos. 1-2, pages 171-174, see entire document.	1-20
Y	HELGESON, E.A. et al. Effects of glucan on mast cell secretion in-vivo. 69th annual meeting of the federation of american societies for experimental biology. 21-26 April 1985, Vol. 44, No. 4, pages 1323, No. 5334.	1-14
Y	KRUEGER-KRASAGAKES, S. et al. Production of interleukin-6 by human mast cells and basophilic cells. Journal of Investigative Dermatology. 01 January 1996, Vol. 106, No. 1, pages 75-79, especially page 76.	3, 5

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 AUGUST 1998	Date of mailing of the international search report 14 OCT 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer MIKE WILSON Telephone No. (703) 308-0000

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11702

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JAFFE, J.S. et al. Human lung mast cell IL-5 gene and protein expression: Temporal analysis of upregulation following IgE-mediated activation. American Journal of Respiratory cell and molecular biology. 1995, Vol. 13, No. 6, pages 665-675, especially page 670.	5
Y	NAIR, A.P.K. et al. A v-H-ras-dependent hemopoietic tumor model involving progression from a clonal stage of transformation competence to autocrine interleukin 3 production. Molecular cell biology. March 1989, Vol. 9, No. 3, pages 1183-1190, especially page 1186.	4
Y,P	US 5,637,463 A (DALTON S. et al.) 10 June 1997, col. 1, line 1.	12
A,P	DVORAK, A.M. New aspects of mast cell biology. International Archives of Allergy and Immunology. 1977, Vol. 114, No. 1, pages 1-9, see entire document.	1-20
Y	NOLTA, J.A. et al. Retroviral vector-mediated gene transfer into primitive human hematopoietic progenitor cells: Effects of mast cell growth factor (MGF) combined with other cytokines. Exp. Hematol. October 1992, Vol. 20, No. 9, pages 1065-1071, especially page 1066.	15-17
Y	DVORAK, A.M. et al. Piecemeal degranulation of mast cells in the inflammatory eyelid lesions of interleukin-4 transgenic mice. Evidence of mast cell histamine release in vivo by diamine oxidase-gold enzyme-affinity ultrastructural cytochemistry. American Society of Hematology. 1994, Vol. 83, No. 12, pages 3600-3601, 3604-3612, especially page 3600.	18-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11702

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/11702

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-14, drawn to methods of identifying molecular components of the mast cell secretory machine and methods of identifying compounds effecting the mast cell secretory machine .
Group II, claim(s) 15-20, drawn to a method of gene therapy and a method of identifying molecular compounds effecting the mast cell secretory machine using genetically altered lower organisms.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The technical feature linking Groups I and II appears to be that they both relate to rab3 genes. However, Oberhauser et al (1994, FEBS, Vol. 339, pp 171-174) teach the roab3 isoforms expressed in peritoneal mast cells. Therefore, the technical linking the inventions of groups I and II does not constitute a special technical feature as defined by PCR Rule 13.2 as it does not define a contribution over the prior art.

The special technical feature of Group I is considered to be a method for determining the molecular components of the mast cell secretory machine.
The special technical feature of Group II is considered to be a method of gene therapy for altering mast cell secretion.

Accordingly, Groups I and II are not so linked by the same or corresponding special technical feature as to form a single general inventive concept.